IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: R. Hutson
Serial No.	:	09/282,239	
Cnfrm. No.	:	8339	Art Unit: 1652
Filed	:	March 31, 1999	
For	:	A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS	

DECLARATION OF MAHENDRA S. RAO, M.D., PH.D. UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, MAHENDRA S. RAO, M.D., Ph.D., pursuant to 37 C.F.R. § 1.132, declare:

1. I received an M.D. (MBBS) degree in Medicine from Bombay University, Bombay India and a Ph.D. degree in Medicine from California Institute of Technology in Pasadena, California.

2. I am a Section Chief for the Stem Cell Unit at the Laboratory of Neuroscience, at NIA (National Institute on Aging), Triad Technology Center, 333 Cassell Drive, Baltimore, MD 21224; an Associate Professor of Neurosciences at Johns Hopkins University School of Medicine, Baltimore, MD 21224; and an Associate Professor at NCBS, Bangalore, India.

3. I am a founder of and shareholder in Q Therapeutics, Inc., 615 Arapeen Drive, Suite 102, Salt Lake City, Utah 84108, which I understand has an exclusive license under the present patent application.

4. I am familiar with the subject matter of the present patent application which I understand is directed to an enriched or purified preparation of human mitotic oligodendrocyte progenitor cells where the cyclic nucleotide phosphodiesterase 2 promoter (i.e. CNP2") is transcriptionally active in all cells of the enriched or purified preparation.

5. I am a co-inventor of U.S. Patent No. 6,361,996 ("'996 Patent"), which I understand has been used as a basis for rejecting claims in the above application. I present this declaration to demonstrate why the subject matter of the '996 Patent is very different from that of the present patent application.

6. The '996 Patent discloses multipotential neuroepithelial stem cells and lineage-restricted astrocyte/oligodendrocyte precursor cells. The astrocyte/oligodendrocyte precursor cells are derived from neuroepithelial stem cells, are capable of self-renewal, and can differentiate into astrocytes and oligodendrocytes but not neurons. The '996 Patent characterizes these cells as "multipotential intermediate precursor cells restricted to glial lineages" (emphasis added)(column 23, lines 1-5). Similarly, my paper Rao, et. al., "Glial-Restricted Precursors are Derived From Multipotential Neuroepithelial Stem Cells," *Devel. Biol.* 188: 48-63 (1997) clearly demonstrates that such A2B5+/NCAM cells are capable of generating both astrocytes and oligodendrocytes and do not appear committed to the oligodendrocyte lineage. The '996 Patent's astrocyte/oligodendrocyte precursor cells are in a less differentiated state than the oligodendrocyte progenitor cells of the present patent application and, therefore, are very different from the cells described in this present application.

7. Differences in the method, time of isolation, and propagation should also be noted. The cells in the present application were derived from the adult brain using a promoter reporter based strategy where the CNP2 promoter directed expression of green fluorescent protein. On the other hand, the astrocyte/oligodendrocyte precursor cells of the '996 Patent were derived from fetal and neonatal tissue using cell surface antigen expression and fluorescence based antibody capture. No strategy of using CNP2 (a cyctoplasmic marker) expression, a CNP2 promoter, or a related promoter reporter strategy is described in the '996 Patent.

8. The '996 Patent is directed to the enrichment of glial progenitor cells from newborn rat brain. Newborns have an abundant population of still-developing oligodendrocyte progenitor cells that may constitute a significant fraction of all of the cells in neonatal brain tissue. Yakovlev, et. al., "A Stochastic Model of Brain Cell Differentiation in Tissue Culture," *J Math Biol.*, 37(1):49-60 (1998)(Appendix 1); Bogler et. al., "Measurement of Time in Oligodendrocyte-type-2

Astrocyte (O-2A) Progenitors is a Cellular Process Distinct from Differentiation or Division," *Dev Biol.*, 162(2):525-38 (1994)(Appendix 2); Raff et. al., "Platelet-derived Growth Factor From Astrocytes Drives the Clock That Times Oligodendrocyte Development in Culture." *Nature* 333(6173):562-65 (1988)(Appendix 3) describe cell cycle changes as glial progenitor cells mature. They showed that adult cells differ in their cell cycle time and the number of divisions before they will become postmitotic. The present patent application discloses this for adult human-derived cells. In addition, adult-derived human oligodendrocyte progenitor cells differentiate as oligodendrocytes and produce myelin much more quickly than do fetal or neonatal oligodendrocyte progenitor cells. In particular, as recently reported in Nunes et al., "Identification and Isolation of Multipotent Neural Progenitor Cells from the Subcortical White Matter of the Adult Human Brain," *Nature Medicine* 9:239-247 (2003) (Appendix 4) and Windrem et al., "Fetal and Adult Human Oligodendrocyte Progenitor Cell Isolates Myelinate the Congenitally Dysmyelinated Brain," *Nature Medicine* 10:93-97 (2004) (Appendix 5), adult-derived oligodendrocyte progenitor cells not only myelinate much more rapidly than do fetal oligodendrocyte progenitors, but they do so more efficiently, with a higher proportion exhibiting effective myelin production, and myelinating a greater number of neuronal axons per donor cell than their fetal-derived counterparts. Adult cells are thus fundamentally more biased towards generating oligodendrocytes, towards maturing to express myelin proteins, and towards myelinating host axons. Moreover, adult cells execute all of these functions, and achieve each of these cellular milestones, much more quickly than fetal cells. As a result, they lend themselves to a very different set of potential clinical targets than fetal or neonatal-derived progenitors, as recently reported in Roy et al., "Progenitor Cells of the Adult Human Subcortical White Matter In: *Myelin Biology and Disorders*, vol. 1. R. Lazzarini, ed. Elsevier:Amsterdam, pp. 259-287 (2004) (Appendix 6). The adult oligodendrocyte progenitor cells of the present application are thus fundamentally different from the fetal or neonatal-derived astrocyte/oligodendrocyte precursor cells of the '996 Patent.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 10/14/04

Mahendra S. Rao MD., Ph.D.

J. Math. Biol. (1998) 37: 49–60

Journal of Mathematical Biology

A stochastic model of brain cell differentiation in tissue culture

A. Y. Yakovlev, M. Mayer-Proschel, M. Noble

Huntsman Cancer Institute, Department of Oncological Sciences, University of Utah, 546 Chipeta Way, Suite 1100, Salt Lake City, UT 84108, USA
e-mail: andrej.yakovlev@genetics.utah.edu

Received: 18 April 1997/Revised version: 30 November 1997

Abstract. The timing of cell differentiation can be controlled both by cell-intrinsic mechanisms and by cell-extrinsic signals. Oligodendrocyte type-2 astrocyte progenitor cells are known to be the precursor cells that give rise to oligodendrocytes. When stimulated to divide by purifed cortical astrocytes or by platelet-derived growth factor, these progenitor cells generate oligodendrocytes *in vitro* with a timing like that observed *in vivo*. The most widely accepted model of this process assumes a cell-intrinsic biological clock that resides in the progenitor cell. The intrinsic clock model originally proposed in 1986 remains as the dominant theoretical concept for the analysis of timed differentiation in this cell lineage. However, the results of a recent experimental study (Ibarrola et al., Developmental Biology, vol. 180, 1–21, 1996) are most consistent with the hypothesis that the propensity of a clone of dividing O-2A progenitor cells initially to generate at least one oligodendrocyte may be regulated by cell-intrinsic mechanisms, but that environmental signals regulate the extent of further oligodendrocyte generation. We propose a stochastic model of cell differentiation in culture to accommodate the most recent experimental findings. Our model is an age-dependent branching stochastic process with two types of cells. The model makes it possible to derive analytical expressions for the expected number of progenitor cells and of oligodendrocytes as functions of time. The model parameters were estimated by fitting these functions through data on the average (sample mean) number of both types of cells per colony at different time intervals from start of experiment. Using this method we provide a biologically meaningful interpretation of the observed pattern of oligodendrocyte generation *in vitro* and its modification in the presence of thyroid hormone.

Key words: Brain cells – Differentiation – Proliferation – Branching process

1 Introduction

It is a striking feature of ontogenic development that particular cell types first appear at precisely regulated moments in the history of the organism and then increase in number over a time period that is very similar in members of the same species. Understanding the biological principles which underly such appropriately timed cell generation is one of the profound challenges in developmental biology.

Any analysis of the timely generation of differentiated cell types must by necessity begin with investigation of the transition from dividing precursor cell to differentiated (and often non-dividing) progeny cell. In order to obtain data that allow one to develop an understanding of the complexities of such differentiation processes, it is necessary to gain access to a wide range of detailed information at a clonal level. Ideally, one should be able to examine multiple clones of dividing precursor cells and to unambiguously distinguish between precursor cells and differentiated progeny. If one is interested in analysis of these processes in cells derived from vertebrates, then experimental studies of this nature must be performed in tissue culture (to enable visualization of cell clones and their development over extended time periods), leading to the further requirement that it be possible to mimic *in vitro* the developmental processes which are thought to occur *in vivo*.

One of the few cellular lineages in which it is possible to obtain the complete collection of data described above is derived from the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell of the central nervous system. It is possible to grow O-2A progenitors cells *in vitro* in such a manner that they divide and generate oligodendrocytes with a timing which recapitulates the timing of normal development *in vivo*. O-2A progenitor cell division can be promoted by purified cortical astrocytes (of a separate glial lineage), and this induction of cell division can also be brought about by platelet-derived growth factor (PDGF) and by the O-2A progenitor mitogen produced by cortical astrocytes (Noble and Murray, 1984; Noble et al., 1988; Pringle et al., 1991; Raff et al., 1988; Bogler et al., 1990). Embryo-derived O-2A progenitor cells induced to divide by PDGF or by type-1 astrocytes will generate oligodendrocytes with a timing that mimics *in vitro* the timing which is seen *in vivo* (Raff et al., 1988).

The initiation of the timely generation of oligodendrocytes within a clonal family of dividing O-2A progenitor cells has for many years been thought to be controlled by a cell-intrinsic biological clock that induces symmetric and synchronous differentiation of all clonally-related O-2A progenitor cells into oligodendrocytes within a relatively short period of time (Temple and Ruff, 1985). According to this model, oligodendrocyte generation is associated with preclusion of the self-renewal process of precursor cells, and thus is in some ways analogous to the limited mitotic life-span expressed by many primary cell types. This hypothesis provided a simple model for the study of temporally regulated differentiation, but it now seems likely that this hypothesis is both incorrect and overly simplistic.

In contrast to the above hypothesis, it was recently discovered that instead of differentiating symmetrically, clonal families of O-2A progenitor may undergo a protracted period of oligodendrocyte generation, during which time the probability of precursor cell self-renewal is regulated predominantly by cell-extrinsic signaling molecules rather than by cell-intrinsic biological clocks (Ibarrola et al., 1996). In other words, it was found that it is possible to distinguish experimentally between the probability that a clone of dividing O-2A progenitor cells will generate at least one oligodendrocyte at an appropriate time *in vitro* and the actual extent of oligodendrocyte generation in that clone. While the latter process was influenced by the presence or absence of exogenous factors (such as thyroid hormone), the former was not.

In respect to the initial generation of oligodendrocytes, it appears that the extent of oligodendrocyte generation within an individual clone may be stochastic. For example, there was no apparent relationship between the number of oligodendrocytes found and the ratio of oligodendrocytes to progenitor cells, excepting that the rare colonies consisting wholly of oligodendrocytes all contained 10 cells or less. In addition, when oligodendrocytes first appeared in cultures of embryonic brain cells the number of oligodendrocytes per colony ranged from 1 to 88, the proportion of oligodendrocytes in heterogeneous colonies ranged from <1% to 81%, and the fractional representation of oligodendrocytes in a colony was not correlated with the number of oligodendrocytes in that colony.

In this paper, we develop a stochastic model which is specifically designed to make quantitative inferences from experimental data on brain cell differentiation *in vitro*.

2 Experimental procedures

Purification of O-2A progenitor cells is described at length by Ibarrola et al. (1996). Purified progenitor cells derived from 7 day old animals were plated at a density of 2500-3000 cells. Cells were fed with PDGF. After plating the culture was scored for the presence of individual cells. Plates with cells in clumps were discarded and not included in the experiment. A fixed number of clones was randomly selected and the cell type composition of each clone was recorded at different times. Cell-types were identified by morphology and cell types were confirmed at the end of each experiment by immunofluorescence using cell type specific antibodies. The design of experiments with thyroid hormone was identical.

3 The model for oligodendrocyte generation in culture

Our model for the stochastic mechanism of O-2A progenitor cell differentiation *in vitro* is a multitype age-dependent branching stochastic process. The model structure is defined by the following set of assumptions.

(i) The process begins with a single progenitor, or type-1, cell cultured at time $t = 0$. This initiator cell may, when it divides, produce two types of cells: progenitor cells of the same type, and oligodendrocytes or type-2 cells. The initiator cell and its descendants are not susceptible to death.

(ii) At the end of the mitotic cycle, every type-1 cell gives rise to two daughter progenitor cells with probability p, and it transforms (differentiates) into an oligodendrocyte with probability $1 - p$. Thus, the probability generating function of the cell progeny is specified as

$$h_1(s) = ps_1^2 + (1 - p)s_2 , \tag{1}$$

where $s = (s_1, s_2)$. The corresponding generating function for type-2 cells is

$$h_2(s) = s_2 , \tag{2}$$

that is, oligodendrocytes neither divide nor die. We do not provide a description of cell death, lest the model be nonidentifiable. The event of cell death is very rare during the first 6 days of observation (Ibarrola et al., 1996), and we take advantage of this fact when estimating the model parameters. The death of oligodendrocytes normally begins on day 7 and its rate increases with time. The contribution of cell death at these late times can be inferred from experimental data by the indirect route.

(iii) The lengths of the mitotic cycle of the initiator cell and its descendants of the same type are independent and identically distributed nonnegative random variables with a common cumulative distribution function $F(t)$. We assume that $F(0) = 0$.

(iv) Progenitor cells, the only migratory cells in the population, do not migrate out of the field of observation.

(v) The usual independence assumptions regarding the evolution of age-dependent branching processes (Jagers, 1975) are adopted.

The model thus specified is a special case of the Bellman-Harris branching process with two types of particles; its asymptotic properties were studied in detail by Jagers (1969, 1975).

Let $Z_i(t)$, $i = 1, 2$, be the number of cells of the ith type at time t. The probability generating functions of $Z_1(t)$ and $Z_2(t)$ can be obtained using general methods of the theory of branching processes. A detailed exposition of these methods can be found in Yakovlev and Yanev (1989). Consider the two-dimentional stochastic process $Z(t) = (Z_1(t), Z_2(t))$ and introduce the generating function

$$\Phi(t, s) = (\Phi_1(t, s), \Phi_2(t, s)) , \tag{3}$$

with the components

$$\Phi_i(t, s) = \sum_k \Pr\{Z(t) = k | Z(0) = e_i\} s^k , \quad i = 1, 2, |s| \le 1 , \tag{4}$$

where $e_1 = (1, 0)$, $e_2 = (0, 1)$, and the summation in (4) is over the set of all points in R^2 with nonnegative integer coordinates. With $h_1(s)$ and $h_2(s)$ given by (1) and (2), the generating functions $\Phi(t, s)$ satisfy the following equations

$$\Phi_1(t, s) = s_1[1 - F(t)] + p\int_0^t \Phi_1^2(t - u, s)\,dF(u) + (1 - p)\int_0^t \Phi_2(t - u, s)\,dF(u)\,, \tag{5}$$

$$\Phi_2(t, s) = s_2\,.$$

Setting $s_1 = z$ and $s_2 = 1$ in (5), it is easy to derive equations for the corresponding marginal generating functions $\varphi_1(t, z) = \Phi_1(t, z, 1)$ and $\varphi_2(t, z) = \Phi_2(t, z, 1)$:

$$\varphi_1(t, z) = z[1 - F(t)] + (1 - p)F(t) + p\int_0^t \varphi_1^2(t - u, z)\,dF(u) \tag{6}$$
$$\varphi_2(t, z) = 1\,.$$

In a similar manner, for $\psi_1(t, z) = \Phi_1(t, 1, z)$ and $\psi_2(t, z) = \Phi_2(t, 1, z)$ we obtain

$$\psi_1(t, z) = 1 - F(t) + p\int_0^t \psi_1^2(t - u, z)\,dF(u) + (1 - p)zF(t) \tag{7}$$
$$\psi_2(t; z) = z\,.$$

Let $M_1(t) = \varphi_1'(t, 1)$ be the expected number of type-1 cells at time t. From (6) it follows that

$$M_1(t) = 1 - F(t) + 2p\int_0^t M_1(t - u)\,dF(u)\,. \tag{8}$$

The expected number of type-2 cells is $M_2(t) = \psi_1'(t, 1)$. Thus we see from (7) that

$$M_2(t) = (1 - p)F(t) + 2p\int_0^t M_2(t - u)\,dF(u)\,. \tag{9}$$

The integral equations (8) and (9) can be generalized to incorporate possible death of type-1 cells (Jagers, 1975). Furthermore they can be solved in closed form (see Athreya and Ney, 1972). Introducing the notation

$$G^{*0}(t) = 1, \qquad G^{*1}(t) = G(t), \qquad G^{*(n+1)}(t) = \int_0^t G^{*n}(t - u)\,dG(u)\,,$$

the solution of (8) is represented as

$$M_1(t) = \sum_{n=0}^{\infty} (2p)^n[F^{*n}(t) - F^{*(n+1)}(t)]\,. \tag{10}$$

Similarly the solution of (9) is given by

$$M_2(t) = (1 - p)\sum_{n=0}^{\infty} (2p)^n F^{*(n+1)}(t)\,. \tag{11}$$

The n-fold convolution F^{*n} of F with itself can be found in an explicit form for some distributions of the mitotic cycle duration. The most popular choice in cell-kinetics studies is the two-parameter gamma distribution (Yakovlev et al., 1977; Nedelman and Rubinow, 1981; Nedelman et al., 1987; Yakovlev and Yanev, 1989, to name a few). We shall proceed from the same choice because this parametric family is quite flexible and reflects a multistage structure of the cell cycle. Some authors (Hartmann et al., 1975; Yakovlev and Zorin, 1988) indicate that the results of kinetic analysis of cell proliferation are usually insensitive to the form of $F(t)$ where absolutely continuous unimodal distributions are concerned.

Suppose that $F(t)$ is specified as the gamma distribution with shape parameter α and scale parameter β. Then we have

$$F^{*n}(t) = \frac{\beta^{\alpha n}}{\Gamma(\alpha n)} \int_0^t x^{\alpha n - 1} e^{-\beta x} dx . \tag{12}$$

To ensure computationally convenient formulas for $M_1(t)$ and $M_2(t)$ it is reasonable to limit possible values of α to the set of positive integers. This constraint has little effect on the accuracy of estimation of the parameter α from experimental data on the mean size of cell clones. For integer α formula (12) reduces to

$$F^{*n}(t) = 1 - e^{-\beta t} \sum_{k=0}^{\alpha n - 1} \frac{(\beta t)^k}{k!} , \tag{13}$$

and we have

$$M_1(t) = e^{-\beta t} \left\{ \sum_{k=0}^{\alpha - 1} \frac{(\beta t)^k}{k!} + \sum_{n=1}^{\infty} (2p)^n \left[\sum_{k=0}^{\alpha(n+1)-1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{\alpha n - 1} \frac{(\beta t)^k}{k!} \right] \right\} , \tag{14}$$

$$M_2(t) = (1 - p) \left[\frac{1}{1 - 2p} - e^{-\beta t} \sum_{k=0}^{\alpha - 1} \frac{(\beta t)^k}{k!} - e^{-\beta t} \sum_{n=1}^{\infty} (2p)^n \sum_{k=0}^{\alpha(n+1)-1} \frac{(\beta t)^k}{k!} \right] . \tag{15}$$

The timing of oligodendrocyte generation *in vitro* has been found to be fundamentally similar to that which occurs *in vivo*. Therefore, it is natural to assume that the population of progenitor cells eventually becomes extinct. In terms of our model this means that the process $Z_1(t)$ is subcritical and we should limit our consideration to the case $p < 0.5$. Then formula (11) implies that $M_2(t)$ is a monotone nondecreasing function and

$$M_2(0) = 0, \qquad \lim_{t \to \infty} M_2(t) = \frac{1 - p}{1 - 2p} . \tag{16}$$

The behavior of $M_2(t)$ is intuitively appealing because in the long run the population of oligodendrocytes is expected to level off to a constant size.

Difficulties emerge when we look more closely at the expected number of progenitor cells. Recalling formula (10) we see that

$$M_1(0) = 1, \qquad \lim_{t \to \infty} M_1(t) = 0 . \tag{17}$$

It also follows from (10) that $M_1(t)$ is a nonincreasing function of time. Indeed, representing the series (10) as

$$\begin{aligned} M_1(t) &= \sum_{n=0}^{\infty} (2p)^n F^{*n}(t) - \sum_{n=0}^{\infty} (2p)^n F^{*(n+1)}(t) \\ &= \sum_{n=0}^{\infty} (2p)^n F^{*n}(t) - F(t) - \sum_{k=0}^{\infty} (2p)^{k+1} F^{*(k+2)}(t) \\ &= 1 - (1-2p)F(t) + \sum_{n=2}^{\infty} (2p)^n F^{*n}(t) - \frac{1}{2p} \sum_{k=0}^{\infty} (2p)^{k+2} F^{*(k+2)}(t) \\ &= 1 - (1-2p)\left[F(t) + \frac{1}{2p} \sum_{n=2}^{\infty} (2p)^n F^{*n}(t) \right], \end{aligned}$$

it is easy to see that $M_1(t)$ is nonincreasing in t whenever $p < 0.5$.

The behavior of $M_1(t)$ appears to be in conflict with experimental data presented in Sect. 4. Since each colony begins with exactly one clonogenic cell at time $t = 0$ one should expect (see Figs. 1 and 2, Sect. 4) that the growth curve for progenitor cells passes through a maximum before it starts decreasing; this is the only pattern consistent with our observations. Clearly, the model must be generalized to allow for the initial increase in the mean number of type-1 cells. The way to do this is through assigning a higher (greater than 0.5) probability of progenitor cell division to initial mitotic cycles. To keep the number of unknown parameters to a minimum, we assume that $p = 1$ for the first N cycles and $p < 0.5$ for the subsequent mitotic cycles. In other words, a progenitor cell acquires the competence for differentiation only after it undergoes a critical number of mitotic divisions. The parameter N is to be estimated from experimental data.

The stepwise change of the probability p can be readily incorporated into the model by introducing N additional types of cells that correspond to the N initial mitotic cycles. We will omit the derivation of the expressions for $M_1(t)$ and $M_2(t)$ given below because it parallels that of formulas (14) and (15). The functions $M_1(t)$ and $M_2(t)$ are given by

$$\begin{aligned} M_1(t) = e^{-\beta t} \Bigg\{ & \sum_{k=0}^{\alpha-1} \frac{(\beta t)^k}{k!} + \sum_{j=1}^{N-1} 2^j \left(\sum_{k=0}^{\alpha(j+1)-1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{\alpha j-1} \frac{(\beta t)^k}{k!} \right) \\ & + 2^N \Bigg[\sum_{k=0}^{\alpha(N+1)-1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{\alpha N-1} \frac{(\beta t)^k}{k!} \\ & + \sum_{n=1}^{\infty} (2p)^n \left(\sum_{k=0}^{\alpha(N+n+1)-1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{\alpha(N+n)-1} \frac{(\beta t)^k}{k!} \right) \Bigg] \Bigg\}, \end{aligned} \tag{18}$$

$$\begin{aligned} M_2(t) = 2^N(1-p) \Bigg[& \frac{1}{1-2p} - e^{-\beta t} \sum_{k=0}^{\alpha(N+1)-1} \frac{(\beta t)^k}{k!} \\ & - e^{-\beta t} \sum_{n=1}^{\infty} (2p)^n \sum_{k=0}^{\alpha(N-n+1)-1} \frac{(\beta t)^k}{k!} \Bigg], \end{aligned} \tag{19}$$

where $N = 1, 2, \ldots, \sum_{j=1}^{0} x_i = 0$, and $0 < p < 0.5$. It immediately follows from (18) and (19) that $M_1(t)$ still satisfies (17) while $M_2(t)$ has the following properties:

$$M_2(0) = 0, \qquad \lim_{t \to \infty} M_2(t) = 2^N \frac{1-p}{1-2p}. \tag{20}$$

4 Inference from experimental data

Equations (19) and (20) provide the basis for estimation of the model parameters by fitting the functions $M_1(t)$ and $M_2(t)$ through data on the average (sample mean) number of both types of cells per colony at different time intervals from start of experiment. We used the least squares method for this purpose. To minimize the sum of squared residuals, use was made of the flexible simplex method (Himmelblau, 1972). The results produced by this nonlinear programming procedure were verified with a program for function minimization included in *MATHEMATICA*.

At a time when we conducted our analysis only the data for $t = 72, 96, 120$ and 144 hours were available, furnishing an opportunity to test the predictive power of the model. In what follows we will refer to these data as Experiment 1. Using the data from Experiment 1 we obtained the following estimates of the model parameters: $\hat{p} = 0.461$, $\hat{N} = 2$, $\hat{\alpha} = 3$, $\hat{\beta} = 0.107$. Thus, the mean, $\tau = \alpha/\beta$, and the standard deviation, $\sigma = \sqrt{\alpha}/\beta$, of the mitotic cycle duration are estimated as $\hat{\tau} = 28$ h and $\hat{\sigma} = 16.2$ h, respectively. The resultant fit is shown in Fig. 1. The model predicts that the mean number of oligodendrocytes tends to a constant level of 27.6 (see formula (20)). Unfortunately, testing this prediction cannot be carried out without the addition of oligodendrocyte survival factors (Noble, 1997), all of which are known to themselves affect the probability of cell differentiation. In our experiments, the cells do not survive that long.

A similar analysis of oligodendrocyte generation in the presence of thyroid hormone (Fig. 2) resulted in the following estimates: $\hat{p} = 0.287$, $\hat{N} = 2$, $\hat{\alpha} = 3$, $\hat{\beta} = 0.137$, whence we have $\hat{\tau} = 21.3$ h and $\hat{\sigma} = 12.6$ h. Hence, thyroid hormone exerts a twofold effect on the cell system under study: it reduces the mean duration of the mitotic cycle of progenitor cells, and it increases the probability of their transformation into oligodendrocytes. The observed dynamics of accumulation of oligodendrocytes is attributable to these mechanisms. It is seen from Fig. 2 that in the presence of thyroid hormone the mean size of the population of oligodendrocytes grows more rapidly attaining a much lower constant level of 6.7 cells predicted by formula (20). At the same time the critical number of initial cycles, N, remains unaltered. These results are in full agreement with qualitative conclusions made by Ibarrola et al. (1996).

An independent experimental study (Experiment 2) was conducted to test the model behavior beyond the observation period chosen in Experiment 1. In

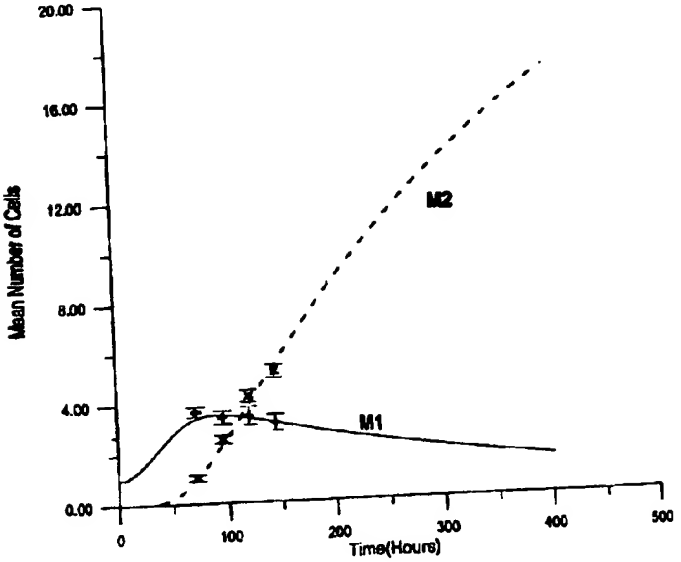

Fig. 1. The dynamic behavior of cell populations in cultures without thyroid hormone (Experiment 1). Solid line represents the expected number of progenitor cells as a function of time; dashed line represents the expected number of oligodendrocytes; + and × are the corresponding experimental data. Bars represent one root square error of estimation at each time point

Experiment 2, data were recorded at $t = 48$, 144 and 192 hours and compared with the corresponding values of $M_1(t)$ and $M_2(t)$ resulted from the analysis of Experiment 1. These results are summarized in Tables 1–4. It is clear from these tables that the model provides a good description of Experiment 2 except the mean number of oligodedrocytes at 192 h (Tables 2 and 4). The observed discrepancy can be attributed to a high rate of cell death at this late time which is not incorporated into the model. As our independent observations show, the proportion of oligodendrocytes surviving by day 8 under *in vitro* conditions is no more than 80%. Yet another factor that may contribute to the discrepancy under discussion is the inter-experimental variation which is difficult to control because the experiments of this kind are very time consuming. One can see from the data for $t = 144$ h that the mean number of oligodendrocyes tends to be smaller in Experiment 2 than in Experiment 1. This tendency may be a manifestation of the higher rate of cell death in Experiment 2 indicated above. However, there is little point in incorporating an explicit description of the process of cell death into the model because the

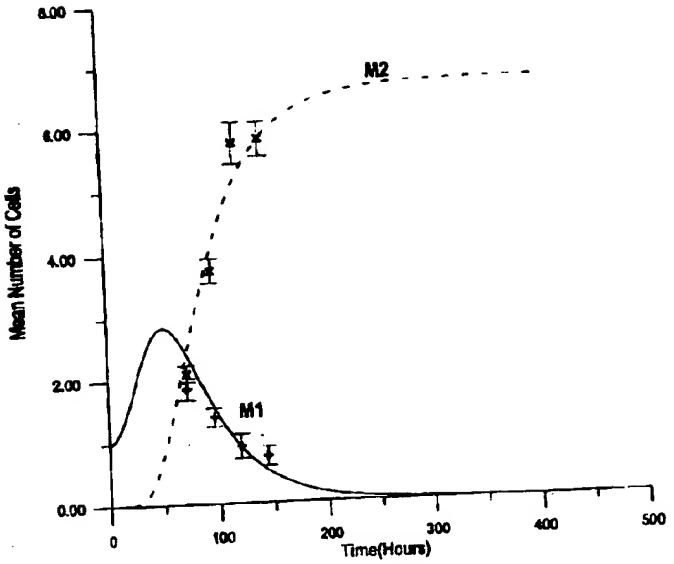

Fig. 2. The dynamic behavior of cell populations in cultures with added thyroid hormone (Experiment 1). Solid line represents the expected number of progenitor cells as a function of time; dashed line represents the expected number of oligodendrocytes; + and × are the corresponding experimental data. Bars represent one root square error at each time point

Table 1. Mean number of O-2A progenitor cells in the absence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_1(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
48	2.68	–	3.4	–	0.24
72	3.40	3.67	–	0.20	–
96	3.54	3.44	–	0.26	–
120	3.40	3.49	–	0.37	–
144	3.20	3.19	2.02	0.33	0.27
192	2.79	–	1.65	-	0.34

available experimental data do not provide sufficient information to identify its quantitative characteristics. The observed overall agreement between the model predictions and experimental data indicates that the postulated stochastic mechanism of oligodendrocyte generation *in vitro* is biologically plausible.

Table 2. Mean number of oligodendrocytes in the absence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_2(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
48	0.18	–	0.27	–	0.08
72	1.00	1.05	–	0.12	–
96	2.38	2.57	–	0.16	–
120	3.95	4.26	–	0.24	–
144	5.50	5.21	2.69	0.25	0.33
192	8.33	–	4.37	–	0.36

Table 3. Mean number of O-2A progenitor cells in the presence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_1(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
48	2.83	–	2.29	–	0.19
72	2.41	1.84	–	0.17	–
96	1.55	1.39	–	0.14	–
120	0.91	0.92	–	0.19	–
144	0.53	0.76	0.92	0.15	0.22
192	0.17	–	0.56	-	0.21

Table 4. Mean number of oligodendrocytes in the presence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_2(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
48	0.68	–	0.54	–	0.10
72	2.41	2.09	–	0.13	–
96	4.06	3.73	–	0.19	–
120	5.16	5.75	–	0.34	
144	5.81	5.81	3.68	0.28	0.29
192	6.40	–	5.42	-	0.37

Acknowledgements We are very grateful to Ms Renlu Gao for computer assistance.

References

Athreya, K. B., Ney, P. E.: Branching processes. Berlin, Heidelberg, New York: Springer-Verlag 1972

Bogler, O., Wren, D., Barnett, S. C., Land, H., Noble, M.: Cooperation between two growth factores promotes extended selfrenewal and inhibits differentiation of oligodendrocyte-type-2 asytocytes (O-2A) progenitor cells. Proc. Natl. Acad. Sci, U.S.A 87, 6368–6372 (1990)

Hartmann, N. R., Gilbert, C. M., Jansson, B., Macdonald, P. D. M., Steel, G. G., Valleron, A. J.: A comparison of computer methods for the analysis of fraction labelled mitoses curves. Cell Tiss. Kinet. 8, 119–124 (1975)
Himmelblau, D. M.: Applied nonlinear programming. New York: McGraw-Hill 1972
Ibarrola, N., Mayer-Proschel, M., Rodriguez-Pena, A., Noble, M.: Evidence for the Existence of at least two timing mechanisms that contribute to oligodendrocyte generation in vitro. Dev. Biol. 180, 1–21 (1996)
Jagers, P: The proportions of individuals of different kinds in two-type populations. A branching process problem arising in biology. J. Appl. Prob. 6, 249–260 (1969)
Jagers, P.: Branching processes with biological applications. New York: Wiley 1975
Nedelman, J., Downs, H., Pharr, P.: Inference for an age-dependent, multitype branching-process model of mast cells. J. Math. Biol. 25, 203–226 (1987)
Nedelman, J., Rubinow, S. I.: Investigation into the experimental kinetic support of the two-state model of the cell cycle. Cell Biophysics 2, 207–231 (1980)
Noble, M.: The oligodendrocyte-type-2 astrocyte lineage: In vitro and in vivo studies on development, tissue repair and neoplasia. In: Isolation, Characterization and Utilization of CNS Stem Cells (Eds Gage, F., Christen, Y.) pp. 101–128. Berlin-Heidelberg: Springer-Verlag 1997
Noble, M., Murray, K.: Purified astrocytes promote the in vitro division of a bipotential glial progenitor cell. EMBO-J 3, 2243–2247 (1984)
Noble, M., Murray, K., Stroobant, P., Waterfield, M. D., Riddle, P.: Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell. Nature 333, 560–562 (1988)
Pringle, N., Collarini, E. J., Hart, I. K., Raff, M. C., Richardson, W. D.: Platelet-derived growth factor in central nervous system gliogenesis. Ann. NY Acad. Sci. 633, 160–168 (1991)
Raff, M. C., Miller, R. H., Noble, M.: A glial progenitor cell that develops in vitro into an astrocytes and oligodendrocyte depending on the culture medium. Nature 303, 390–396 (1988)
Temple, S., Raff, M. C.: Differentiation of a bipotential glial progenitor cell in single cell microculture. Nature 313, 223–225 (1985)
Yakovlev, A. Y., Yanev, N. M.: Transient processes in cell proliferation kinetics. Berlin, Heidelberg, New York: Springer Verlag 1989
Yakovlev, A. Y., Zorin, A. V.: Computer simulation in cell radiobiology. Berlin, Heidelberg, New York: Springer Verlag 1988
Yakovlev, A. Y., Zorin, A. V., Isanin, N. A.: The kinetic analysis of induced cell proliferation. J. Theor. Biol. 64, 1–25 (1977)

DEVELOPMENTAL BIOLOGY 162, 525–538 (1994)

Measurement of Time in Oligodendrocyte-Type-2 Astrocyte (O-2A) Progenitors Is a Cellular Process Distinct from Differentiation or Division

OLIVER BÖGLER*†[1] AND MARK NOBLE*

*Ludwig Institute for Cancer Research, Middlesex Hospital Branch, 91 Riding House Street, London W1P 8BT; and †Molecular Neurobiology Laboratory, Salk Institute for Biological Studies, P.O. Box 85800, San Diego, California 92186-5800

Accepted December 23, 1993

When stimulated by platelet-derived growth factor (PDGF), oligodendrocyte-type-2 astrocyte (O-2A) progenitors derived from perinatal rat optic nerves undergo a limited number of cell divisions before clonally related cells synchronously and symmetrically differentiate into nondividing oligodendrocytes. The duration of this mitotic period is thought to be controlled by a cell-intrinsic biological clock. Thus, in the presence of PDGF, the measurement of time by the biological clock is intimately coupled to the control of division and differentiation. In contrast, O-2A progenitors grown in the presence of PDGF plus basic fibroblast growth factor (bFGF) divide indefinitely in the absence of differentiation and so do not exhibit a limited period of division. We have tested whether growth in PDGF plus bFGF alters the duration of the limited period of division O-2A progenitors exhibit in response to PDGF alone. Accordingly, O-2A progenitors were grown in the presence of PDGF plus bFGF for varying lengths of time, before being switched to conditions that promote timed differentiation (PDGF but not bFGF). Increasing duration of culture in PDGF plus bFGF led to a gradual shortening of the period for which O-2A progenitors were subsequently responsive to PDGF alone, until eventually all cells differentiated without dividing after switching. In contrast, a short exposure to bFGF was not sufficient to cause a similar alteration in the pattern of differentiation. These results indicate that O-2A progenitors prevented from undergoing timed differentiation nevertheless retain the ability to measure elapsed time, implying that the biological clock in this cell type can be uncoupled from differentiation. Furthermore, they demonstrate that the biological clock does not impose an absolute limit on the number of divisions that an O-2A progenitor can undergo. In contrast with existing hypotheses, our observations suggest that the molecular mechanism that controls timed differentiation must consist of at least two components, with the clock itself being in some manner distinct from mechanisms that limit cell division and/or directly regulate differentiation. © 1994 Academic Press, Inc.

INTRODUCTION

Several observations in a variety of cell types suggest that measurement of elapsed time by cells is closely linked to the initiation of terminal differentiation. For example, hematopoietic stem cells generate erythroid cells which switch from production of fetal hemoglobin to adult hemoglobin after the passage of a seemingly preprogrammed length of time (Zanjani *et al.*, 1979; Wood *et al.*, 1985). Similarly, the number of divisions a fibroblast can undergo before terminally differentiating into a senescent cell appears to be preprogrammed or limited (reviewed in Goldstein, 1990). The measurement of elapsed time also seems to play a major controlling role in the timing of differentiation of glial precursor cells of the central nervous system (CNS)[2] into oligodendrocytes (Abney *et al.*, 1981; Temple and Raff, 1986; Noble *et al.*, 1988; Raff *et al.*, 1988; reviewed in Groves *et al.*, 1991, and Noble, 1991). The apparent coupling of the measurement of elapsed time to differentiation in these systems raises the question of whether these two processes are mechanistically distinct.

Understanding the regulatory mechanisms that make it possible for cells to differentiate according to an intrinsic schedule has been a subject of considerable inter-

[1] To whom correspondence should be addressed at present address: Ludwig Institute for Cancer Research, San Diego Branch, 9500 Gilman Drive, La Jolla, CA 92093-0660.

[2] Abbreviations used: Ast, astrocyte coculture; Ast/P, astrocyte coculture with additional exogenous PDGF; Ast/PF, astrocyte coculture with exogenous PDGF plus bFGF; bFGF, basic fibroblast growth factor; CNS, central nervous system; DMEM-BS, Dulbecco's Modified Eagle's Medium modified according to Bottenstein and Sato, 1979; DMEM-FCS, DMEM containing fetal calf serum; E19, Embryonic Day 19; ECM, extracellular matrix; O-2A, oligodendrocyte-type-2 astrocyte; P7, Postnatal Day 7; PDGF, platelet-derived growth factor; SV40 T, simian virus 40 large tumor antigen.

0012-1606/94 $6.00

est. Hypotheses about the nature of cellular timing mechanisms are as varied as the terms used to describe the phenomenon itself. Thus the terms "biological clock," "developmental clock," "time-measuring ability of cells," "finite mitotic life span," and other similar phrases have been used to describe phenomena in which a cell-intrinsic timing or measuring system appears to control the timing of differentiation. Due to the lack of knowledge about what is being measured by biological clocks, we describe this phenomenon as the measurement of elapsed time (see for example Orgel, 1973). This should be taken only as indicating that the passage of time is associated with the changes in the behavior of cells and is not meant to suggest that cells measure time in the same way that mechanical clocks do. It has been suggested, for example, that "biological time [is] equivalent to trains of specific physical or chemical events, which is a very different concept than that of an intrinsic clock based on sidereal or calendar time" (Finch, 1990). In the instances of particular interest here, it is clear that the duration of the period to be measured is determined at least several (and sometimes many) cell divisions before the differentiation event is itself observable. Most investigations of this phenomenon have been carried out on fibroblasts (which enter a nondividing senescent state after a limited number of cell divisions) and the range of hypotheses advanced to explain the limited mitotic life span of these cells includes random accumulation of cellular damage (Szilard, 1959; Orgel, 1973; Goldstein, 1990), telomere shortening (Harley *et al.*, 1990), changes in negative-growth regulatory genes (Weinberg, 1993), and progression through a genetic program (Orgel, 1973; Bayreuther *et al.*, 1988a,b; Goldstein, 1990). One could equally imagine that timed differentiation is caused by a steady increase, over a number of cell divisions, in the amount of a differentiation-inducing activity which determines cellular phenotype only after passing a threshold. Alternately, the steady decrease in the amount of some activity absolutely required for cell division could trigger the timed cessation of division.

All of the hypothetical mechanisms proposed to explain the workings of biological clocks appear to share the common feature of predicting that when the measuring process has been completed, differentiation (and, in at least some instances, cessation of division) follows necessarily. For example, if the period of division were limited by the accumulation of cellular damage or telomere shortening, then these events could not occur in cells stimulated to divide beyond their "normal" limit, for these events should by themselves be sufficient to limit cell division. Similarly, if the functioning of the biological clock relied simply on the buildup of a transcription factor to a level required to induce differentiation, then accumulation of such a factor should not occur in cells prevented from differentiating.

One experimental system that can be used to analyze the relationship among the measurement of elapsed time, the cessation of cell division, and the onset of differentiation is the timed differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells into oligodendrocytes. O-2A progenitors from embryonic rat CNS can be cultured in such a manner as to generate the first oligodendrocytes at a time equivalent to the day of birth, when the first oligodendrocytes appear *in vivo* (Abney *et al.*, 1981; Raff *et al.*, 1985, 1988). This appropriately scheduled differentiation in culture requires the presence of cortical astrocytes (Raff *et al.*, 1985) or purified platelet-derived growth factor (PDGF) (Raff *et al.*, 1988), an O-2A progenitor mitogen secreted by cortical astrocytes (Noble and Murray, 1984; Noble *et al.*, 1988; Richardson *et al.*, 1988). For example, in the presence of PDGF, optic nerve cultures from Embryonic Day 18 (E18) rats would generate the first oligodendrocytes after 3 days *in vitro*, while cultures from E19 rats would do so after 2 days (Raff *et al.*, 1988). That the scheduled differentiation of oligodendrocytes relied on the ability of O-2A progenitors to measure elapsed time was implied by the observation that clonally related O-2A progenitors generally ceased proliferating and differentiated within one division of each other, even if grown in separate tissue culture dishes after their first division (Temple and Raff, 1986). Synchronous differentiation of clonally related O-2A progenitors is also observed if cells are grown in chemically defined medium containing PDGF (Noble *et al.*, 1988; Raff *et al.*, 1988). Thus, existing observations suggest that the appropriately timed generation of oligodendrocytes relies on a cell-intrinsic clock that resides within the O-2A progenitor and measures cell divisions or some other parameter (Temple and Raff, 1986).

In contrast to the behavior of O-2A progenitors stimulated to divide with PDGF, the appropriately timed generation of oligodendrocytes does not occur if cultures of optic nerve cells are grown in the absence of mitogen or are treated simultaneously with PDGF and basic fibroblast growth factor (bFGF). In the absence of mitogen, O-2A progenitors differentiate rapidly and prematurely into oligodendrocytes without dividing (Raff *et al.*, 1983; Noble and Murray, 1984; Temple and Raff, 1985). The diametrically opposite result is obtained if O-2A progenitors are grown in the presence of PDGF plus bFGF, a condition in which continuous division of O-2A progenitors is maintained in the absence of differentiation (Bögler *et al.*, 1990; Groves *et al.*, 1993). These latter results indicate that O-2A progenitors are not intrinsically limited to a relatively small number of divisions

before differentiating. Rather, such a limit represents a pattern of division and differentiation observed only when cells are grown specifically in the presence of PDGF.

The ability to promote continuous division of O-2A progenitors in the absence of differentiation by treatment with PDGF plus bFGF has offered us the opportunity to study in more detail the relationship between the measurement of time by a biological clock and the onset of differentiation and cessation of division. Our data show that prolonged exposure to PDGF plus bFGF alters the behavior O-2A progenitors subsequently exhibit in response to PDGF alone. It is also shown that a brief exposure to bFGF, in the continued presence of PDGF, did not alter the timing of differentiation of O-2A progenitors into oligodendrocytes. These results suggest that the measurement of time still occurs under conditions in which division continues indefinitely in the absence of differentiation. This implies that the measurement of elapsed time can be separated mechanistically from the mechanisms that control the onset of differentiation or the cessation of cell division.

MATERIALS AND METHODS

Analysis of Small Populations and Clones of Optic Nerve Cells

Primary optic nerve cultures and purified cortical astrocytes were established as described previously (McCarthy and De Vellis, 1980; Raff *et al.*, 1983; Noble and Murray, 1984; Raff *et al.*, 1985). For the period of culture in the presence of PDGF plus bFGF optic nerve cells were seeded in poly-L-lysine-coated 25-cm^2 flasks at 200,000 to 300,000 cells per flask in DMEM-BS, a chemically defined medium (a modification of the medium described by Bottenstein and Sato, 1979; Bögler *et al.*, 1990). Bulk optic-nerve-cell cultures were given 10 ng/ml of recombinant human PDGF A-chain homodimer [Chiron Corporation (a kind gift of Dr. C. George-Nascimento) or Promega] and recombinant human bFGF (Boehringer-Mannheim or Promega) each day and half the medium was changed every other day. Once a week the cells were passaged by trypsinization in calcium- and magnesium-free DMEM containing 200 μg/ml EDTA and 6000 U/ml trypsin, followed by trypsin inhibitor (added 1 part in 3.5; 0.52 mg/ml soybean trypsin inhibitor, 0.04 mg/ml bovine pancreas DNase, and 3 mg/ml BSA fraction V in DMEM; Sigma). An aliquot of cells was removed for analysis at the times indicated under the Results section. The remainder of the cells were returned to bulk culture as above.

For analysis of small populations of optic nerve cells (Fig. 1), cocultures were established with cortical astrocytes, which produce PDGF (Noble *et al.*, 1988) and maximize optic nerve cell viability (Temple and Raff, 1986; Raff *et al.*, 1988; see also Barres *et al.*, 1992). As the age of the cortical astrocyte cultures could conceivably affect the behavior of O-2A progenitors (see Lillien and Raff, 1990, for one such phenomenon), fresh cultures of astrocytes were prepared according to identical schedules for each analysis. Astrocyte cultures were equivalent to P21 at the time that optic nerve cells were plated on them, an age when O-2A progenitor division and differentiation are still occurring *in vivo* (Miller *et al.*, 1985). Furthermore, these astrocyte cultures were made in the same way as those used previously to establish that astrocytes make PDGF (Noble *et al.*, 1988; Raff *et al.*, 1988). Monolayers of 20,000 cortical astrocytes per poly-L-lysine-coated coverslip (Chance Propper No. 1, 13 mm) were cultured in 0.5 ml of DMEM containing 10% fetal calf serum (DMEM-FCS; Gibco BRL), which was changed 2 days later. After 3 days the cultures were irradiated with 2000 rads of X rays, washed once, and then fed with 0.5 ml DMEM-BS. One day later 100 μl of filter-sterilized DMEM-BS conditioned for 48 hr by cortical astrocytes (from a flask of the same cortex preparation that had not been passaged) was added to each well shortly before the optic nerve cells. Five hundred optic nerve-derived cells were delivered to the supernatant of these cultures in 10 μl. Approximately 50 to 100 cells were observed after 1 day on the coverslips covering part of the bottom of the well (Fig. 1). Half of the medium was replaced every other day, and growth factors were added daily.

For analysis of O-2A progenitor clones (Fig. 2) 1000 cortical astrocytes were plated in 10 μl of DMEM-FCS per Terasaki microwell and the plates incubated upside down for the first 48 hr, so that the astrocytes attached predominantly to the sides of the wells. This facilitated observation of the optic nerve cells subsequently plated into the microwells. These cultures were treated identically to those on coverslips described above, except: (a) instead of changing the medium after 2 days, 10 μl of DMEM-FCS was added; (b) they were not washed after irradiation before the medium was replaced with 10 μl of DMEM-BS; and (c) no conditioned medium was added separately, but the final dilutions of optic nerve cells were made in astrocyte-conditioned medium. A variety of dilutions of optic nerve-derived cells, designed to deliver between 1 and 10 cells per 10 μl per well, were plated into the Terasaki wells. The next day, and every 2 days after that, 10 μl of the medium was removed and the cultures were observed, and 10 μl DMEM-BS containing 10 μg/ml PDGF was replaced. After oligodendrocytic differentiation was judged to be complete on the basis of the morphology of visible cells, the cells

TABLE 1
bFGF ATTACHES TO THE MATRIX OF E19 OPTIC NERVE CULTURES

	E19 cells grown for first 24 hr in:	ECM incubated with:	Indicator cells cultured in:	% O-2A lineage cells GalC$^+$ after 4 days *in vitro*
1	—	—	PDGF	46.9 ± 3.2
2	—	—	PDGF + bFGF	3.3 ± 0.4
3	PDGF	PDGF	PDGF	57.4 ± 2.3
4	PDGF	PDGF	PDGF + bFGF	5.1 ± 0.8
5	bFGF	—	PDGF	4.0 ± 0.4
6	bFGF	—	PDGF + bFGF	3.2 ± 0.9
7	—	bFGF	PDGF	3.7 ± 0.4
8	—	bFGF	PDGF + bFGF	4.3 ± 0.6

Note. Exposure of E19 optic nerve cultures to bFGF either before or after lysis results in an extracellular matrix that inhibits the differentiation of O-2A progenitors in conjunction with PDGF. E19 optic nerve cultures were grown in the absence of growth factor (rows 1, 2, 7, and 8) in the presence of PDGF (rows 3 and 4) or in the presence of bFGF (rows 5 and 6) before they were lysed by hyposmotic shock. The ECM was then incubated for 24 hr in the absence of growth factor (rows 1, 2, 5, and 6) in PDGF (rows 3 and 4) or bFGF (rows 7 and 8) before indicator cells derived from 7-day-old optic nerve were plated onto the ECM and grown for a further 4 days in the presence of PDGF (odd-numbered rows) or PDGF plus bFGF (even-numbered rows). Data are presented as means ± SEM of three experiments.

were prepared for immunocytochemistry. The time required for oligodendrocytic differentiation to appear complete varied from culture to culture and depended on the size of the clones. The smallest clones (Fig. 2C) appeared to have differentiated after 3 days or less and were prepared for immunocytochemistry at that time. The largest clones were prepared for immunocytochemistry 10 days after optic nerve cells were plated into the microwells. Observations made before and after immunocytochemistry were compared, and only if they were sufficiently similar so that it appeared beyond reasonable doubt that a group of cells was derived from the single O-2A progenitor observed on Day 1 was the clone allowed into the data set shown in Fig. 2.

Analysis of Embryonic Optic Nerve Cultures

Initial experiments showed that E19 cultures that were given bFGF only on Day 1 behaved identically to those that received bFGF every day over a 10-day period: no oligodendrocytes appeared (data not shown; all cultures received PDGF every day). One possibility was that bFGF was persisting in these cultures due to interactions with the extracellular matrix (ECM; for review see Gospodarowicz *et al*, 1987; see also Lillien and Raff, 1990). To investigate this directly, the interaction of bFGF with the ECM of E19 optic nerve cultures was studied. E19 optic nerve cells were plated on coverslips and cultured in the absence of factors or in the presence of either PDGF or bFGF (10 ng/ml each) for 24 hr. These cultures were then subjected to hyposmotic lysis after 24 hr to generate an adherent cell lysate which consists predominantly of ECM (as in Lillien and Raff, 1990). Coverslips were then washed once with PBS and twice with DMEM-BS, incubated for 24 hr in DMEM-BS, or in DMEM-BS containing either PDGF or bFGF (20 ng/ml), and washed twice in DMEM-BS. Indicator cells from P7 optic nerve cultures maintained in PDGF plus bFGF for 2 days were then plated onto the ECM and grown for a further 4 days in the presence of PDGF or PDGF plus bFGF (10 ng/ml each) before they were prepared for immunocytochemistry. If bFGF was present either before lysis, or after lysis but before the indicator cells were added, differentiation was inhibited as much as if bFGF was present after the indicator cells were added (Table 1, all indicator cell cultures received PDGF). In contrast, if the cultures saw either no growth factors before the indicator cells were plated, and then only PDGF, or if they saw only PDGF throughout, considerable differentiation occurred (Table 1). Therefore, it appears that bFGF became strongly attached to the ECM of these cultures and was able to affect the differentiation of O-2A progenitors in that attached form. In order to be sure to eliminate any bFGF attached to the ECM after the first 24 hr of culture, E19 optic nerve cultures were established in bulk (as above) and passaged by trypsinization after 24 hr. Cells were then plated on poly-L-lysine-coated coverslips at a density of 10,000 cells/coverslip in DMEM-BS. Cultures received either PDGF or PDGF plus bFGF throughout, or PDGF plus bFGF for the first 24 hr and then PDGF on a daily basis, at 10 ng/ml each. Approximately half the DMEM-BS was changed every 2 days, and cells were prepared for immunocytochemistry at the times indicated.

PDGF Receptor Analysis

For the PDGF receptor analysis shown in Figs. 4 and 5, optic nerve cultures were established in bulk and treated as described earlier. Aliquots of cells removed for analysis were plated directly onto poly-L-lysine-coated coverslips at a density of 3000 cells per coverslip. They were cultured for 48 hr in the presence of PDGF or PDGF plus bFGF (10 ng/ml each) and prepared for immunocytochemistry.

Immunocytochemistry

The antibodies used were monoclonal anti-galactocerebroside (anti-GalC) antibody (Ranscht *et al*, 1982) and monoclonal antibody A2B5 (Eisenbarth *et al*, 1979).

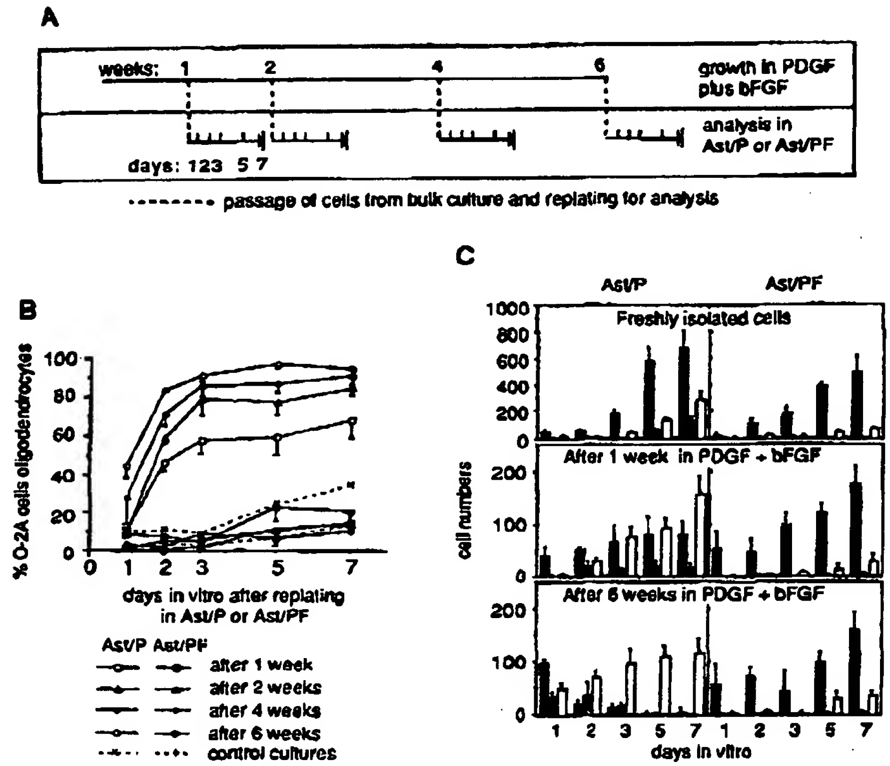

FIG. 1. Culture in PDGF plus bFGF leads to a gradual increase in the proportion of O-2A progenitors that no longer divide in response to Ast/P. (A) A schematic representation of the experimental design. Optic nerve cultures from 7-day-old rats were established in bulk for the period of culture in PDGF plus bFGF. Cells were passaged weekly, an aliquot was removed for analysis after 1, 2, 4, and 6 weeks, and the remaining cells were returned to bulk culture. Analysis was performed in coculture with cortical astrocytes and exogenous PDGF (Ast/P). (B) The percentage of O-2A lineage cells that were GalC$^+$ oligodendrocytes in optic nerve cell/astrocyte cocultures is shown graphed against time in coculture. Cocultures received either PDGF (Ast/P) or PDGF plus bFGF (Ast/PF). Cells were either derived from PDGF plus bFGF-treated bulk cultures (solid lines; mean ± SD of three or four coverslips per point) or were freshly isolated from optic nerve (dashed lines; control cells were analyzed in parallel with each experimental curve and the data pooled; mean ± SEM). For control cells only, the number of oligodendrocytes found in Ast/PF cultures on Day 1 was subtracted from all the control-cell data to allow a direct comparison with experimental cultures which contained almost no oligodendrocytes at the time of plating. As no new oligodendrocytes are generated in Ast/PF this number most closely resembles the number of oligodendrocytes initially plated. Only half of the error bars are shown for clarity, and if no error bars are shown then the SD or SEM was smaller than the radius of the plot symbol. Data is from one representative experimental series of three. (C) The numbers of A2B5$^+$GalC$^-$ O-2A progenitors (black bars), A2B5$^+$GalC$^+$ immature oligodendrocytes (stippled bars), or A2B5$^-$GalC$^+$ oligodendrocytes (white bars) in optic nerve cell/astrocyte cocultures are shown, graphed against time in coculture. Optic nerve cells were freshly isolated (top panels) or had been cultured in PDGF plus bFGF for 1 week (middle panels) or for 6 weeks (bottom panels). During the time shown parallel cultures received either PDGF only (Ast/P) or PDGF plus bFGF (Ast/PF). None of the cultures showed significant oligodendrocytic differentiation if cultured in PDGF plus bFGF: the number of oligodendrocytes shown in the right panels is similar to the numbers seen in astrocyte cultures to which no optic nerve cells were added. Data are means ± SD of three or four coverslips and are part of one representative experimental series of three.

The A2B5 monoclonal antibody specifically labels O-2A lineage cells in these cultures and is an IgM (Raff *et al.*, 1983), while anti-GalC, an IgG3, specifically labels oligodendrocytes (Raff *et al.*, 1978). Fluorescein- and rhodamine-conjugated second-layer antibodies against monoclonal antibodies were from Southern Biotechnology and were used at a dilution of 1:100. Fluorescein-conjugated anti-rabbit antibodies were from Tioga (USA) and were used at 1:200. Standard immunofluorescence to identify cells of the O-2A lineage was performed as described (Raff *et al.*, 1988; Noble and Murray, 1984; Bögler *et al.*, 1990). Anti-PDGF receptor immunofluorescence was done as follows. Cultures were rinsed in phosphate-buffered saline, fixed for 10 min in 4%

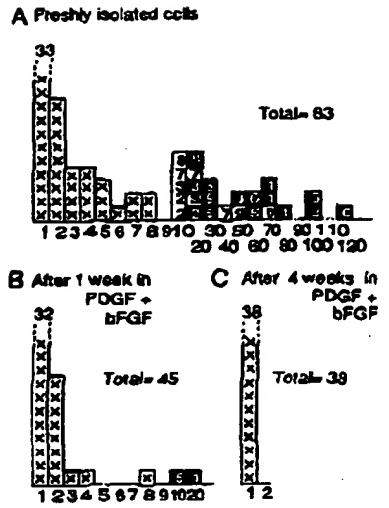

FIG. 2. Prolonged culture in PDGF plus bFGF leads to a reduction in the size of clones derived from individual O-2A progenitors. A stem and leaf plot (as in Temple and Raff, 1986) showing the reduction in clone size in the presence of astrocytes and PDGF, after switching from culture in PDGF plus bFGF. Each 'x' or number represents one clone. To obtain the number of cells in a given clone either read the number below the column in which the 'x' is or add the number in the graph (units) to the number under the column (tens). For example in the upper panel there are three clones of five cells and five clones that are between 10 and 20 cells consisting of 12, 12, 13, 17, and 18 cells. The total number of clones analyzed under each condition is indicated. The experimental results are from two separate bulk cultures that were analyzed after 1 and 4 weeks of culture in PDGF plus bFGF; data from freshly isolated P7 rat optic nerve cultures analyzed in parallel with each experiment were pooled. Clones shown in black writing on white ground consisted entirely of GalC$^+$ oligodendrocytes; clones shown as white writing on black ground contained some A2B5$^+$ GalC$^-$ O-2A progenitors, and these cells always comprised less than a third of the total cells, and typically about 10%.

paraformaldehyde, and then exposed sequentially to the following in Hepes-buffered Hanks' balanced salt solution (Imperial Laboratories) containing 5% newborn calf serum and 5% goat serum (both from GibcoBRL): 25 min A2B5 and anti-GalC, 25 min anti-IgG-biotin, 25 min streptavidin-coumarin (Molecular Probes, Oregon) and anti-IgM-fluorescein, 10 min 0.1% v/v Triton X-100, 45 min anti-PDGF receptor antibody (1:500; UBI), two 5-min washes, 30 min anti-rabbit fluorescein, two 5-min washes. Cultures were viewed on a Zeiss Axiphot microscope equipped with phase contrast and epi-uv illumination and selective filters for fluorescein, rhodamine, and coumarin.

Immunoprecipitation and Western Blot Analysis

Cultures of NIH 3T3 mouse fibroblasts, Rat-2 fibroblasts, and O-2A progenitors were harvested before becoming confluent by rinsing in PBS and scraping in 600 μl Ripa buffer (150 m*M* NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 m*M* Tris, pH 8.0) containing 0.5 m*M* PMSF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 40 μg/ml bestatin, and 1 μg/ml aprotinin. Insoluble material was removed by centrifugation, and the supernatant was immunoprecipitated by the addition of 20 μl of R7 anti-PDGF receptor antiserum (Eriksson *et al*, 1992) and 60 μl of protein A–Sepharose *CL4B* (Pharmacia) and incubating on ice for 60 min. The immunoprecipitate was washed three times in 500 μl of Ripa buffer, and an equal volume of 2× sample buffer was added. The precipitates were boiled, separated on an acrylamide gel, and blotted to Immobilon P membrane (method described in Harlowe and Lane, 1988). The immunoblot was preblocked for 4 hr in blotto (5% nonfat dry milk in phosphate-buffered saline, 0.02% azide), exposed overnight to R7 antibody (1:400), washed extensively and exposed to ^{125}I-donkey anti-rabbit antibodies for 1 hr, followed by several washes, and exposed to X ray film.

RESULTS

O-2A progenitors dividing and differentiating in the presence of cortical astrocytes or PDGF exhibit three observable behaviors from which the functioning of their cell-intrinsic clock can be inferred. The first behavior is the ability of populations of O-2A progenitors to continue to generate new oligodendrocytes for a prolonged period of time, a process that is dependent upon ongoing O-2A progenitor division and differentiation (Noble and Murray, 1984; Temple and Raff, 1986; Noble *et al*, 1988; Raff *et al*, 1988). In contrast, if O-2A progenitors are no longer able to divide, oligodendrocyte numbers rapidly increase initially and then show no further change when all the O-2A progenitors have differentiated (assuming that the fairly consistent rate of differentiation of individual cells (e.g., Raff *et al*, 1984) is itself not changed by the experimental manipulations). The second behavior that can be observed is the generation of clones of oligodendrocytes derived from a single O-2A progenitor. The size of the clone depends on the length of the limited period of division elicited by PDGF and so reflects the period of time that remained to be measured by the founding O-2A progenitor before differentiation was initiated (Temple and Raff, 1986). The third behavior is exhibited by populations of O-2A progenitors derived from embryonic optic nerve. Such populations give rise to the first oligodendrocytes *in vitro* at a time equivalent to the day of birth (Noble *et al*, 1988; Raff *et al*, 1988). This last behavior is the measure most likely to indicate whether the biological clock in O-2A progenitors is functioning appropriately.

The effect of preventing differentiation while stimulating division for varying periods of time has been experimentally determined for all three of these behaviors. The experimental design used for the analyses of the first two behaviors (experiments reported in Figs. 1 and 2) was to grow O-2A progenitors (derived from the optic nerves of 7-day-old rats) under conditions that prevented differentiation (PDGF plus bFGF) and to switch cells after different durations of culture to conditions that promoted timed differentiation (PDGF but not bFGF). The ability of these cells to divide and differentiate was then compared to that of freshly isolated control O-2A progenitors. Accordingly, optic nerve cells were established in bulk culture and given PDGF plus bFGF daily. Cultures were passaged weekly, and aliquots of cells were removed and replated in the presence of purified cortical astrocytes for analysis (see Fig. 1A). Coculture with cortical astrocytes was used as astrocytes make PDGF and promote timed differentiation of O-2A progenitors (Noble *et al.*, 1988; Richardson *et al.*, 1988) as well as maximizing O-2A lineage cell viability (Temple and Raff, 1986; Barres *et al.*, 1992), thereby allowing the analysis of small populations of cells or even individual clones. Astrocyte-optic nerve cocultures received either no additional factors, PDGF, or PDGF plus bFGF (referred to as Ast, Ast/P, or Ast/PF, respectively) and were analyzed by immunocytochemistry after 1, 2, 3, 5, and 7 days. As expected from the fact that astrocytes make PDGF, we observed no differences between cultures that received no additional factors (data not shown) and those that received PDGF alone and present data only on cells that were switched to Ast/P.

Populations of freshly isolated O-2A progenitors cultured in the presence of PDGF exhibit both O-2A progenitor self-renewal by division and oligodendrocyte generation by differentiation (Noble and Murray, 1984; Noble *et al.*, 1988; see also Wren *et al.*, 1992). In PDGF-treated cultures of optic nerve cells, the extent of O-2A progenitor self-renewal is related to the proportion of O-2A progenitors with some time remaining in their limited period of division, and so have not undergone timed differentiation. The capacity of O-2A progenitor self renewal to continue for prolonged periods in populations of cells is thought to reflect a heterogeneity in the duration of the mitotic period remaining in different clones of the starting population (Temple and Raff, 1986). As a consequence one cannot measure the timed differentiation of O-2A progenitors into oligodendrocytes in postnatal optic nerve cultures as a single endpoint. The changing numbers of O-2A progenitors and oligodendrocytes in a population analyzed at various time points can be used, however, to give some indication of the amount of time that remains to be measured by the

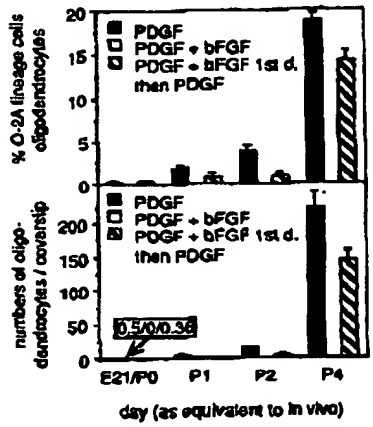

FIG. 3. Transient exposure to PDGF plus bFGF does not perturb the appearance of the first oligodendrocytes in embryonic optic nerve cultures. Data shows the percentage of O-2A lineage cells that were GalC$^+$ oligodendrocytes (top) or the average number of oligodendrocytes seen per coverslip (bottom) in cultures derived from E19 optic nerve. The average number of oligodendrocytes per coverslip at the time equivalent to E21/P0 is shown as numbers (PDGF:0.5/PDGF plus bFGF:0/PDGF plus bFGF for first day, then PDGF: 0.36), as the bars are too small to be seen. Cultures were established in bulk for the first 24 hr and then passaged and replated on coverslips. They received either PDGF throughout, PDGF plus bFGF throughout, or PDGF plus bFGF for the first 24 hr and then PDGF only. Differentiation state was analyzed by immunocytochemistry at 2, 3, 4, and 6 days after dissection, shown as days equivalent to *in vivo*. Data are presented as means ± SEM of three experiments.

O-2A progenitors in that population. If, on average, a large amount of time remains in the limited period of division that precedes timed differentiation, then one would observe the continuous generation of both O-2A progenitors and oligodendrocytes. In contrast, if all the O-2A progenitors have completed the measurement of time and reached the end of their period of division, they would be expected to differentiate rapidly into oligodendrocytes and to show little sign of self-renewal.

Culture of O-2A Progenitors in PDGF Plus bFGF Leads to a Gradual Reduction in the Duration for Which They are Subsequently Mitotically Responsive to PDGF Alone

Analysis of populations of O-2A progenitors that had been switched from culture in PDGF plus bFGF to Ast/P showed a dramatic increase in the proportion of O-2A lineage cells that were oligodendrocytes after the first 3 days in Ast/P, when compared to freshly isolated O-2A progenitors (Fig. 1B). Furthermore, the proportion of the starting population that differentiated into oligodendrocytes during the first 3 days of culture in Ast/P

TABLE 2
O-2A PROGENITORS THAT ARE NO LONGER MITOTICALLY RESPONSIVE TO PDGF EXPRESS PDGF RECEPTORS

Time of culture in PDGF plus bFGF	Percentage of O-2A progenitors that were PDGF receptor⁻	
	PDGF	PDGF + bFGF
Control cells	96.1 ± 1.1	95.9 ± 1.1
1 week	96.0 ± 1.4	96.8 ± 0.9
2 weeks	98.1 ± 0.5	98.0 ± 0.7
4 weeks	99.6 ± 0.3	98.7 ± 0.5
6 weeks	97.9 ± 0.8	98.3 ± 0.5

Note. The percentage of O-2A progenitors that were PDGF receptor⁻ after various times of culture in the presence of PDGF plus bFGF followed by a further 2 days of culture in PDGF or PDGF plus bFGF. For culture in the presence of PDGF plus bFGF cells were treated as described for the experiments shown in Figs. 1 and 2. Aliquots of cells were removed after 1, 2, 4, and 6 weeks and replated at a density of 3000 cells per coverslip. They were treated with either PDGF or PDGF plus bFGF and prepared for immunocytochemistry after 2 days. They were labeled with anti-PDGF receptor antibodies, as well as A2B5 and anti-GalC antibodies. The proportion of A2B5⁺ GalC⁻ O-2A progenitors that were also labeled by the anti-PDGF receptor antibodies is shown. Data is mean ± SEM for between 6 and 10 coverslips from two separate experiments.

correlated well with the duration of prior culture in PDGF plus bFGF (Fig. 1B): the longer cells had been grown in the presence of PDGF plus bFGF the greater the proportion of O-2A lineage cells that differentiated into oligodendrocytes after 3 days in Ast/P. Three days after switching cells to Ast/P the rate of increase of the percentage of oligodendrocytes slowed, either because the rate of O-2A progenitor self-renewal became similar to the rate of oligodendrocyte generation (after 1, 2, and 4 weeks in PDGF plus bFGF) or because there were almost no O-2A progenitors left at this time (after 6 weeks in PDGF plus bFGF). As expected, all cultures in Ast/PF showed little increase in the proportion of O-2A lineage cells that were oligodendrocytes as under these conditions O-2A progenitor division occurred in the absence of oligodendrocyte differentiation (Fig. 1B).

Examination of the data shown in Fig. 1B in terms of cell numbers suggested that after 6 weeks in PDGF plus bFGF, no significant amount of O-2A progenitor division occurred in Ast/P (Fig. 1C, bottom left panel shows no change in the sum of the bar heights; the culture conditions prevent any significant cell death: see Barres *et al*, 1992). In contrast, freshly isolated populations of O-2A progenitors, or cells switched after 1 week of culture in PDGF plus bFGF, showed a combination of O-2A progenitor self-renewal and oligodendrocyte generation in Ast/P (Fig. 1C, left panels). The behavior of O-2A progenitors switched to Ast/P after only 1 week of culture in PDGF plus bFGF was intermediate between that of the cells grown for 6 weeks and freshly isolated cells: although some O-2A progenitor division occurred (as indicated by an increase in cell number), a larger proportion of cells in the 1 week pre-treated culture became oligodendrocytes within 3 days than in cultures of freshly isolated cells (compare Fig. 1C left panels). As expected, all Ast/PF cultures showed O-2A progenitor division and no significant oligodendrocyte generation over the time points examined (Fig. 1C).

Cells differentiating from an A2B5⁺GalC⁻ O-2A progenitor into an A2B5⁻GalC⁺ oligodendrocyte are transiently A2B5⁺GalC⁺, and the proportion of O-2A lineage cells that occupy this compartment at any given time is related to the rate of differentiation of the population as a whole. Examination of this A2B5⁺GalC⁺ compartment also shows that pretreatment with PDGF plus bFGF is associated with an increase in the number of differentiating cells seen at early time points (Fig. 1C, stippled bars). Again, 6 weeks of prior culture in PDGF plus bFGF had a more marked effect than 1 week of prior culture (Fig. 1C, left panels). After 6 weeks in the presence of PDGF plus bFGF, very few A2B5⁺GalC⁻ cells were found in these culture after the 3-day time point (Fig. 1C). In contrast, A2B5⁺GalC⁺ cells were found on all days examined in cultures grown in the presence of PDGF plus bFGF for 1 week before switching to Ast/P. It is interesting to note, however, that significant numbers of such cells were seen in these cultures several days before their presence in cultures derived from freshly isolated cells. Thus, these data also indicate that the yield of oligodendrocytes within a given time period of growth in Ast/P is increased by prior culture in PDGF plus bFGF and further indicate that the rate of differentiation itself is probably not altered from the figure of 3 days reported in earlier studies (Raff *et al*, 1984).

Culture of O-2A Progenitors in PDGF Plus bFGF Leads to a Stepwise Reduction in the Size of the Oligodendrocyte Clones Subsequently Generated in Response to PDGF Alone

Clonal analysis allows direct measurement of the number of cell divisions that the founding cell of a clone and its progeny underwent. In the case of O-2A progenitors dividing in response to PDGF, clone size depends on the length of the limited mitotic period that precedes timed differentiation, assuming that no significant amount of cell death occurred (Temple and Raff, 1986). Therefore, clones derived from O-2A progenitors that

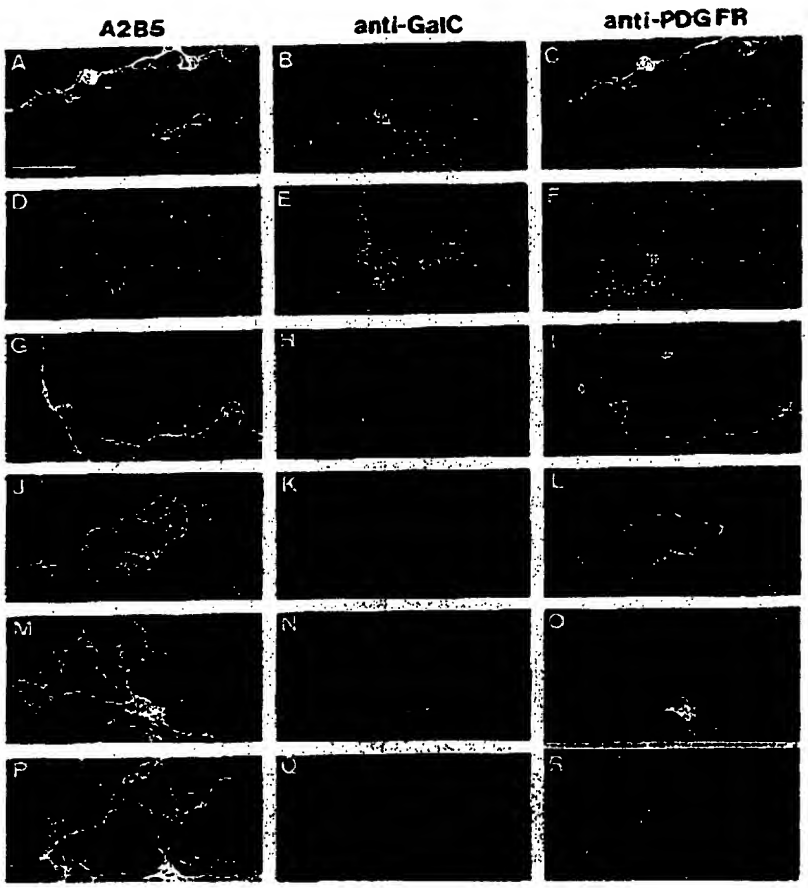

FIG. 4. O-2A progenitors cultured in PDGF plus bFGF for 6 weeks followed by 2 days in PDGF continue to express PDGF receptors. Three-color immunocytochemistry of optic nerve cultures using monoclonal A2B5 (via rhodamine) and anti-GalC (via coumarin) and polyclonal anti-PDGF receptor antibodies (via fluorescein). Cells were either derived directly from P7 optic nerve (A to I) or had undergone 6 weeks of preculture in PDGF plus bFGF (J to R). They were cultured for 2 days in the presence of PDGF alone (A to F and J to O) or in the presence of PDGF plus bFGF (G to I and P to R). $A2B5^-GalC^-$ O-2A progenitors were PDGF $receptor^+$ under all conditions (A to C; G to L; P to R). In contrast, $A2B5^-GalC^+$ oligodendrocytes such as the one indicated by the arrow in B, were negative for the PDGF receptor. D to F and M to O show $A2B5^+GalC^+$ immature oligodendrocytes that are PDGF $receptor^+$. In the control cultures that were derived from P7, optic nerve cells that were PDGF $receptor^+$, but did not label with either A2B5 or anti-GalC, were common (short arrows in I). These cells were probably pial meningeal cells. The scale bar in A is 30 μm.

had been grown in PDGF plus bFGF for varying periods of time, or had been freshly isolated from optic nerve, were analyzed in insulin containing coculture with cortical astrocytes (Noble *et al.*, 1988; Raff *et al.*, 1988; Barres *et al.*, 1992), following the method of Temple and Raff (1986). Cultures were observed the day after optic nerve cells were placed in microculture and every other day after that. When oligodendrocytic differentiation was judged to be complete by cell morphology (after between 3 and 10 days, depending on how many rounds of division occurred) cultures were prepared for immunocytochemistry to confirm the differentiation state of the cells and to facilitate counting of the cells.

Clones derived from freshly isolated O-2A progenitors showed a wide range of sizes as expected (Fig. 2A; Temple and Raff, 1986). In contrast, after 4 weeks in PDGF plus bFGF all of the 38 O-2A progenitors analyzed generated clones of 1 oligodendrocyte (Fig. 2C), suggesting that these O-2A progenitors were no longer induced to divide by PDGF when grown in single-cell microculture. None of the founding cells was seen to divide and all differentiated within 3 days of plating

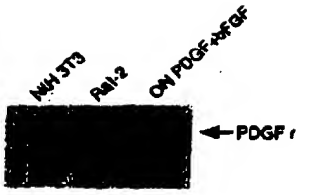

FIG. 5. O-2A progenitors cultured for 7 weeks in PDGF plus bFGF express PDGF-α receptor. Anti-PDGF-α receptor Western blot of anti-PDGF-α receptor immunoprecipitates of Rat-2, NIH 3T3 fibroblasts, and an optic nerve culture that had been grown for 7 weeks in the presence of PDGF plus bFGF. The position of the PDGF-α receptor is indicated, and these bands migrated at approximately 180 kDa.

into the microwells. O-2A progenitors that had been first grown for a week in PDGF plus bFGF showed behavior that was intermediate between that of freshly isolated cells and cells that had been in the presence of PDGF plus bFGF for 4 weeks (Fig. 2B). The largest clone derived from freshly isolated cells consisted of 120 cells, implying that the founder O-2A progenitor of this clone underwent at least seven divisions. After 1 week in PDGF plus bFGF clones of 21 cells or fewer were obtained, suggesting that O-2A progenitors underwent a maximum of five divisions after switching to Ast/P. It should be noted that some of the clones shown in Fig. 2 contained A2B5$^+$GalC$^-$ O-2A progenitors at the time that the cultures were fixed (white letters on black background). These cells had appeared to be oligodendrocytes when they were observed live under the phase-contrast microscope as they had a more complex, branched morphology than typical bipolar O-2A progenitors. As in the course of oligodendrocytic differentiation, morphology changes before antigenic phenotype (Noble and Murray, 1984; Raff *et al.*, 1985; Temple and Raff, 1986) and is generally associated with a cessation of division (Small *et al.*, 1987; Noble *et al.*, 1988), it is most likely that these cells were at an early stage of differentiation. The presence of a few A2B5$^+$GalC$^-$ multipolar O-2A progenitors in some clones is consistent with previous observations (Temple and Raff, 1986).

A Short Exposure to bFGF Does Not Alter the Timing of the Appearance of the First Oligodendrocytes in PDGF-Treated Embryonic Optic Nerve Cultures

Analysis of the behavior of small populations (Fig. 1) and clones (Fig. 2) suggested that culture in PDGF plus bFGF led to a gradual shortening of the period of time during which O-2A progenitors would subsequently divide in response to Ast/P. One possible explanation is that cells registered the passing of time in the presence of PDGF plus bFGF and that exposure to bFGF in the presence of PDGF did not prevent the measurement of time in cultures of postnatal optic nerve. An alternative explanation would be that any exposure to bFGF dramatically increases the yield of oligodendrocytes produced within the first few days of subsequent growth in Ast/P. To test this possibility we used the sensitive assay for the correct functioning of the clock provided by the timed appearance of oligodendrocytes in cultures of embryonic optic nerve. The first oligodendrocytes appear in embryonic optic nerve cultures treated with PDGF at a time equivalent to birth, and invariably do so within a 24-hr window (Raff *et al.*, 1988). Therefore, we exposed embryonic optic nerve cultures transiently to bFGF (in the continued presence of PDGF) and followed the appearance of oligodendrocytes.

Transient exposure to bFGF in the continued presence of PDGF did not alter the time of appearance of the first oligodendrocytes in embryonic optic nerve cultures from that observed in control cultures that received PDGF throughout the experiment (Fig. 3). However, slightly fewer oligodendrocytes appeared over the course of the experiment in cultures that transiently received bFGF, both in terms of cell numbers and proportion of O-2A lineage cells. This raises the possibility that short-term exposure to bFGF increased the number of divisions that some clones of cells underwent, as has been suggested by others (McKinnon *et al.*, 1990). Cells that received either PDGF or PDGF plus bFGF throughout behaved as expected from previous studies: in PDGF alone the first oligodendrocytes were generated after 2 days (Fig. 3; Raff *et al.*, 1988), while in PDGF plus bFGF no oligodendrocytes were generated (Fig. 3; Bögler *et al.*, 1990).

O-2A Progenitors That Are Mitotically Unresponsive to PDGF Continue to Express PDGF-α Receptors

One possible explanation for the observation that prolonged culture in the presence of PDGF plus bFGF led to an inability of some or all O-2A progenitors to subsequently respond to Ast/P is that the levels of PDGF-α receptor declined. In order to investigate this possibility, we asked whether long-term cultures of O-2A progenitors grown in the presence of PDGF and bFGF continued to express PDGF-α receptors. We also tested whether O-2A progenitors grown for various periods of time in PDGF plus bFGF and then switched to the presence of PDGF alone for 2 days expressed PDGF-α receptors. Bulk optic nerve cultures derived from P7 optic nerve were established as for previous experiments. Cells were removed from PDGF plus bFGF after 1, 2, 4, and 6 weeks and analyzed by PDGF receptor immunocytochemistry after a further 2 days, either in

the presence of PDGF plus bFGF or in PDGF alone (Table 2 and Fig. 4). This time point was chosen as it represents a compromise between allowing the maximum time for the levels of receptor to decline if it were no longer being synthesized and retaining enough A2B5$^+$GalC$^-$ O-2A progenitors present in all the cultures to be able to analyze a reasonably sized population of cells.

Greater than 95% of A2B5$^+$GalC$^-$ O-2A progenitors showed a clear reaction with anti-PDGF receptor antibodies in all cultures examined, suggesting that they expressed PDGF-α receptor (Table 2 and Fig. 4) despite being refractory to the mitogenic effects of PDGF. In contrast, A2B5$^-$ GalC$^+$ oligodendrocytes were not labeled by the anti-PDGF receptor antibodies in any cultures (Fig. 4A to 4C). In both the control and experimental populations that received only PDGF, A2B5$^+$ GalC$^+$ immature oligodendrocytes that were also PDGF receptor$^+$ were seen, suggesting that at least in some cases PDGF-α receptor levels did not decline until after differentiation was already in progress.

The cells that remained from the bulk culture of one of the above experiments after 6 weeks were returned to culture in the presence of PDGF plus bFGF for a further 7 days before being harvested for analysis by immunoprecipitation and Western blotting with an antiserum specific for the PDGF-α receptor (Eriksson *et al*, 1992). As can be seen in Fig. 5 this optic nerve culture, which contained almost only O-2A progenitors, expressed levels of PDGF-α receptor similar to NIH 3T3 or Rat-2 fibroblasts.

DISCUSSION

When grown in the presence of PDGF, O-2A progenitors derived from optic nerves of perinatal rats undergo a limited period of division, at the end of which they switch from division to differentiation. The duration of this period of division is thought to be regulated by a cell-intrinsic clock. We examined whether O-2A progenitor behavior changed with elapsed time when cells were grown for varying periods of time under conditions that stimulate division and prevent differentiation. It was found that O-2A progenitors grown in the presence of PDGF plus bFGF, which together prevent differentiation, gradually became refractory to the mitogenic effect of subsequent culture in Ast/P in the absence of bFGF. The longer the period in PDGF plus bFGF, the greater the proportion of O-2A progenitors that differentiated without dividing after having been switched to Ast/P. Eventually, after 6 weeks in PDGF plus bFGF, almost all the O-2A progenitors differentiated without dividing in Ast/P. In addition, O-2A progenitors precultured in PDGF plus bFGF gave rise to smaller clones of oligodendrocytes, after switching to single cell culture in Ast/P, than did freshly isolated cells grown in parallel single cell microculture. After 1 week in PDGF plus bFGF the maximum clone size observed after switching to Ast/P was 21 cells compared to 120 cells seen in the largest clone derived from freshly isolated O-2A progenitors. Exposure to both mitogens for 4 weeks was sufficient to produce a population of O-2A progenitors that no longer divided after switching to Ast/P in single cell microculture. In contrast, a short exposure to PDGF plus bFGF did not result in any similar shortening of the period of division of O-2A progenitors after switching to PDGF alone.

We suggest that the simplest explanation of our results is that O-2A progenitors cultured in the presence of PDGF plus bFGF measure time and retain a memory of the fact that they have exceeded the number of divisions that they would normally undergo in the presence of PDGF alone. While it appears that this continued functioning of the biological clock was able to bring cells to the brink of differentiation, it was not sufficient to cause a cessation of cell division or to induce differentiation. These results represent an extension of previous studies of this phenomenon, in that the measurement of time by O-2A progenitors had previously only been observed as the timed switching of cells from a program of self-renewal by division to a program of differentiation, in the presence of PDGF (Noble and Murray, 1984; Temple and Raff, 1986; Noble *et al*, 1988; Raff *et al*, 1988). From these studies it was not possible to determine whether the clock is part of the mechanisms that regulate cell division and differentiation or is separate from them. The demonstration that the measurement of time apparently occurs under conditions where division continues in the absence of differentiation allows us to examine this issue.

Predictions about the behavior of the clock under conditions in which cells divide but do not differentiate differ depending on whether it is a separate and independent mechanism. If the clock were an integral part of either the cell division or differentiation machinery, it would follow that the clock would be inoperative under the growth conditions we examined. In detail, if the clock worked by limiting the number of cell divisions, for example by the loss of an activity required for execution of the cell cycle, then conditions under which this limit is removed would prevent the measurement of time. Similarly, if the clock operated by inducing differentiation after a set period of time, conditions which prevent differentiation would destroy the cell's ability to measure time. Our observations appear to rule out both of these groups of potential mechanisms, as our

data suggest that the measurement of time is ongoing under conditions that induce continued proliferation in the absence of differentiation. Instead, we would suggest that our results are more consistent with the hypothesis that the measurement of time is performed by a separate cellular mechanism that can interact with the mechanisms controlling division and/or differentiation, yet is distinct from these mechanisms.

It should be noted that our experiments do not address the questions of whether cell division is required for the functioning of the clock or whether it is the number of cell divisions that are being counted. In order to examine these issues it would be necessary to analyze the ability of cells to measure elapsed time in the absence of cell division. Therefore, while our hypothesis states that the clock is separate from the cell division machinery (i.e., does not function by imposing an irrevocable limit on the number of cell divisions), it does not state that the clock does not require cell division to function.

Previous analysis of O-2A progenitors in single cell culture has led to the estimate that a maximum of eight rounds of cell division occur in response to stimulation by purified astrocytes (Temple and Raff, 1986), which appear to exert their mitogenic effects through PDGF (Noble *et al.*, 1988; Raff *et al.*, 1988; Richardson *et al.*, 1988). This estimate is at odds with our finding that growth for between 4 and 6 weeks in PDGF plus bFGF is required for O-2A progenitors to reach the point at which they are unresponsive to Ast/P (Figs. 1 and 2). O-2A progenitors grown in PDGF, or in PDGF plus bFGF, appear to have similar cell cycle times of approximately 20 hr (Noble *et al.*, 1988; Bögler *et al.*, 1990). At such a rate of division, at least 28 divisions (4 weeks; see Fig. 2) would be required to exhaust the measurement of time in the presence of PDGF plus bFGF.

One explanation for this difference between our observations and those of Temple and Raff (1986) would be that exposure to bFGF alters the clock by extending the period of time to be measured. This possibility has been raised in the context of a model for the clock whereby the measurement of time is ascribed to a reduction in the level of PDGF-α receptors, in a study where it was observed that bFGF could up-regulate the amount of PDGF-α receptor expressed by O-2A progenitors (McKinnon *et al.*, 1990). This possibility would not be inconsistent with our observation that a short exposure of embryonic optic nerve-derived O-2A progenitors to bFGF was associated with a slower production of oligodendrocytes, although the time of their first appearance was not changed (Fig. 3). However, other data from our study show that exposure to bFGF (in the presence of PDGF) for 1 week or longer leads to a reduction in the subsequent period of division in response to PDGF, suggesting that exposure to bFGF does not inevitably lead to an extension in the clock.

Another possible explanation for the discrepancy between our results and those of Temple and Raff (1986) is that the experimental conditions under which the measurement of time is examined can affect the result. When we analyzed individual O-2A progenitors as opposed to small populations, we observed that a shorter period of previous culture in PDGF plus bFGF resulted in the complete absence of division: for small population analysis 6 weeks was required, while for single cell analysis only 4 weeks sufficed (compare Figs. 1 and 2). This would suggest that any measure of the length of the PDGF responsive period derived from observations in single cell microculture may lead to an underestimate when compared to experiments using small populations. It is not clear whether these differences are due to interactions between O-2A progenitors, changes in the ratio of astrocytes to optic nerve cells, or other factors.

O-2A progenitors that differentiate under the control of the clock lose the ability to divide in response to PDGF before any overt phenotypic changes occur (Temple and Raff, 1986; Noble *et al.*, 1988; Raff *et al.*, 1988). Oligodendrocytes are however not truly postmitotic as they retain the ability to divide in response to bFGF for a period of time in culture (Eccleston and Silberberg, 1985; Saneto and De Vellis, 1985; Noble *et al.*, 1988; Bögler *et al.*, 1990; Mayer *et al.*, 1993). It has, therefore, appeared probable that a key event in triggering timed differentiation in O-2A progenitors is the selective loss of mitotic responsiveness to PDGF. This has seemed unlikely to be due to a loss of PDGF receptors, as at least 50% of newly formed oligodendrocytes express detectable levels of PDGF receptors (Hart *et al.*, 1989b). Furthermore, the PDGF receptors on immature oligodendrocytes are able to transmit signals: PDGF is capable of inducing a rise in intracellular calcium (Hart *et al.*, 1989a) as well as the expression of the proto-oncogenes c-fos and c-jun (Hart *et al.*, 1992). In addition, PDGF can transiently act as a survival factor for oligodendrocytes (Barres *et al.*, 1992) and elevate protein levels of the transcription factor SCIP/Tst-1/Oct-6 in immature oligodendrocytes (O. Bögler, A. Entwistle, R. Kuhn, G. Lemke, and M. Noble, unpublished observation). Our observation that O-2A progenitors that have become refractory to the mitogenic effect of PDGF after prolonged culture in PDGF plus bFGF, but continue to express easily detectable levels of PDGF receptor, is therefore in agreement with the work of others. The possibility that changes too subtle to be discerned with

either anti-PDGF receptor antibodies (this study) or radiolabeled PDGF (Hart *et al.*, 1989b) underlie timed differentiation cannot, however, be excluded at present.

Recent studies conducted on fibroblast populations suggest that the type of phenomenon we have observed is not restricted to O-2A progenitor cells. These experiments have been performed on fibroblast populations derived from H-2K^b-tsA58 transgenic mice (Jat *et al.*, 1991), which can be induced to express the immortalizing tsA58 mutant of SV40 large T antigen immediately after dissection by growth of cells in the presence of interferon at 33°C. Thus, these cells can be exposed to a stimulus that allows them to continue dividing beyond their normal limited mitotic life span from the beginning of their *in vitro* growth, in analogy with the exposure of O-2A progenitors to PDGF plus bFGF. Fibroblasts derived from H-2K^b-tsA58 transgenic mice are only conditionally immortal, in that switch to growth in interferon-free medium at 39.5°C turns off function of the large T antigen, again in analogy with the switch of O-2A progenitors from growth in PDGF plus bFGF to growth in Ast/P. It was shown previously that inactivation of a conditional immortalizing stimulus in long-term rodent fibroblast cultures was associated with the cessation of growth and an accumulation of cells in the G1 and G2/M phases of the cell cycle, suggesting that the removal of active large T antigen led to the recapitulation of senescence (Grove and Cristofalo, 1977; Jat and Sharp, 1989). The more recent studies on fibroblasts derived from H-2K^b-tsA58 transgenic mice have indicated that in these cells the normal mitotic life span is measured despite the presence of large T antigen. Thus, cells switched to nonpermissive conditions after various numbers of passages under immortalizing conditions have a shorter remaining mitotic life span than control cells, with the extent of remaining mitotic life span being related directly to the length of time the cells were first grown under immortalizing conditions. Moreover, cells switched after the normal mitotic life span has elapsed rapidly cease cell division and become senescent (Ikram *et al.*, in press). These results suggest that cells prevented from undergoing senescence by the presence of an immortalizing oncogene retain cellular memory of having passed the number of cell divisions after which they would have ordinarily senesced, implying that the mechanism that regulates the onset of senescence measures time in the presence of an oncogene. The timing mechanisms that regulate senescence and oligodendrocyte differentiation therefore appear to be at least superficially similar in that they remain active when not being able to regulate the onset of the processes they normally control. The extent to which such processes are indeed controlled by similar mechanisms will only be established when the molecular basis of timed differentiation is understood.

We suggest that the data we have presented establish that the biological clock of O-2A progenitor cells is an autonomous mechanism distinct from the mechanisms which allow cell division or promote cell-type-specific differentiation. An elucidation of the molecular nature of the clock may be facilitated by the ability to generate cells which are no longer able to measure time, for example by prolonged culture of O-2A progenitors in PDGF plus bFGF or rodent fibroblasts harboring a temperature-sensitive SV40 large T antigen. Such cells could be used as targets for attempts to reconstitute the measurement of time and so may lead to the identification of the molecules involved.

We thank Drs. Nancy Papalopulu, Andrew Groves, Parmjit Jat, Gus Wolswijk, Chris Kintner, Barbara Barres, Sue Barnett, Paris Ataliotis, Jack Price, John Heath, and Annette Thomas for interesting discussions and helpful comments on this work. We thank Dr. Carl-Henrik Heldin for his kind gift of anti-PDGF receptor antibody. O.B. thanks Drs. Gerry Weinmaster and Peter Maycox for help with immunoprecipitation and Western blotting. O.B. would like to thank Dr. Greg Lemke for allowing the experiments shown in Figs. 4 and 5 to be completed in his laboratory and gratefully acknowledges the support of an EMBO Long Term Fellowship during this part of the work.

REFERENCES

Abney, E. R., Bartlett, P. F., and Raff, M. C. (1981). Astrocytes, ependymal cells, and oligodendrocytes develop on schedule in dissociated cell cultures of embryonic rat brain. *Dev. Biol.* 83, 301–310.

Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyvodic, J. T., Richardson, W. D., and Raff, M. C. (1992). Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 70, 31–46.

Bayreuther, K., Rodemann, H. P., Francz, P. I., and Maier, K. (1988a). Differentiation of fibroblast stem cells. *J. Cell Sci. (Suppl.)* 10, 115–130.

Bayreuther, K., Rodemann, H. P., Hommel, R., Dittmann, K., Albiez, M., and Francz, P. I. (1988b). Human skin fibroblasts in vitro differentiate along a terminal cell lineage. *Proc. Natl. Acad. Sci. USA* 85, 5112–5116.

Bögler, O., Wren, D. R., Barnett, S. C., Land, H., and Noble, M. D. (1990). Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of O-2A progenitor cells. *Proc. Natl. Acad. Sci. USA* 87, 6368–6372.

Bottenstein, J. E., and Sato, G. H. (1979). Growth of a rat neuroblastoma cell line in serum free supplemented medium. *Proc. Nat. Acad. Sci. USA* 76, 514–517.

Eccleston, P. A., and Silberberg, D. R. (1985). Fibroblast growth factor is a mitogen for oligodendrocytes in vitro. *Dev. Brain Res.* 21, 315–318.

Eisenbarth, G. S., Walsh, F. S., and Nirenberg, M. (1979). Monoclonal antibody to plasma membrane antigen of neurons. *Proc. Nat. Acad. Sci. USA* 76, 4913–4917.

Eriksson, A., Siegbahn, A., B., W., Heldin, C. H., and Claesson-Welsh, L. (1992). PDGF alpha- and beta-receptors activate unique and common signal transduction pathways. *EMBO J.* 11, 543–550.

Finch, C. E. (1990). "Longevity, Senescence and the Genome," Univ. of Chicago Press, Chicago and London.

Goldstein, S. (1990). Replicative senescence: The human fibroblast comes of age. *Science* 249, 1129–1133.

Gospodarowicz, D., Neufeld, G., and Schweigerer, L. (1987). Fibroblast growth factor: Structural and biological properties. *J. Cell. Physiol. (Suppl.)* 5, 15–26.

Grove, G. L., and Cristofalo, V. J. (1977). Characterization of the cell cycle of cultured human diploid cells: Effect of aging and hydrocortisone. *J. Cell. Physiol.* 90, 415–422.

Groves, A. K., Bögler, O., Jat, P. S., and Noble, M. (1991). The cellular measurement of time. *Curr. Opin. Cell Biol.* 3, 224–229.

Groves, A. K., Barnett, S. C., Franklin, R. J. M., Crang, A. J., Mayer, M., Blakemore, W. F., and Noble, M. (1993). Repair of demyelinated lesions by transplantation of purified O-2A progenitor cells. *Nature* 362, 453–455.

Harley, C. B., Futcher, A. B., and Greider, C. W. (1990). Telomeres shorten during aging of human fibroblasts. *Nature* 345, 458–460.

Harlow, E., and Lane, D. (1988). "*Antibodies: A Laboratory Manual.*" Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hart, I. K., Richardson, W. D., Bolsover, S. R., and Raff, M. C. (1989a). PDGF and intracellular signaling in the timing of oligodendrocyte differentiation. *J. Cell Biol.* 109, 3411–3417.

Hart, I. K., Richardson, W. D., Heldin, C.-H., Westermark, B., and Raff, M. C. (1989b). PDGF receptors on cells of the oligodendrocyte-type-2 astrocyte (O-2A) cell lineage. *Development* 105, 595–603.

Hart, I. K., Richardson, W. D., and Raff, M. C. (1992). PDGF increases the expression of fos and jun in newly formed oligodendrocytes that have become resistant to the mitogenic effect of PDGF. *Glia* 6, 310–313.

Ikram, Z., Norton, T., and Jat, P. S. (1994). The biological clock that measures the finite mitotic lifespan of mouse embryo fibroblasts continues to function in the presence of SV40 large T antigen. *Proc. Natl. Acad. Sci. USA*, in press.

Jat, P. S., and Sharp, P. A. (1989). Cell lines established by a temperature-sensitive simian virus 40 large-T-antigen gene are growth restricted at the nonpermissive temperature. *Mol. Cell. Biol.* 9, 1672–1681.

Jat, P. S., Noble, M. D., Ataliotis, P., Tanaka, Y., Yannoutsos, N., Larsen, L., and Kioussis, D. (1991). Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse. *Proc. Natl. Acad. Sci. USA* 88, 5096–5100.

Lillien, L. E., and Raff, M. C. (1990). Analysis of the cell–cell interactions that control type-2 astrocyte development in vitro. *Neuron* 4, 525–534.

Mayer, M., Bögler, O., and Noble, M. (1993). The inhibition of oligodendrocytic differentiation of O-2A progenitors caused by basic fibroblast growth factor is overridden by astrocytes. *Glia* 8, 12–19.

McCarthy, K. D., and De Vellis, J. (1980). Preparation of separate astrocyte and oligodendrocyte cultures from rat cerebral tissue. *J. Cell Biol.* 85, 890–902.

McKinnon, R. D., Matsui, T., Dubois-Dalcq, M., and Aaronson, S. A. (1990). FGF modulates the PDGF-driven pathway of oligodendrocytic development. *Neuron* 5, 603–614.

Miller, R. H., David, S., Patel, R., Abney, E. R., and Raff, M. C. (1985). A quantitative immunohistochemical study of macroglial cell development in the rat optic nerve: *In vivo* evidence for two astrocytic lineages. *Dev. Biol.* 11, 35–41.

Noble, M. (1991). Points of controversy in the O-2A lineage: Clocks and type-2 astrocytes. *Glia* 4, 157–164.

Noble, M., and Murray, K. (1984). Purified astrocytes promote the in vitro division of a bipotential glial progenitor cell. *EMBO J.* 3, 2243–2247.

Noble, M., Murray, K., Stroobant, P., Waterfield, M. D., and Riddle, P. (1988). Platelet derived growth factor promotes the division and motility, and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell. *Nature* 333, 560–562.

Orgel, L. E. (1973). Aging of clones of mammalian cells. *Nature* 243, 441–445.

Raff, M. C., Mirsky, R., Fields, K. L., Lisak, R. P., Dorfman, S. H., Silberberg, D. H., Gregson, N. A., Liebowitz, S., and Kennedy, M. (1978). Galactocerebroside: A specific cell surface antigenic marker for oligodendrocytes in culture. *Nature* 274, 813–816.

Raff, M. C., Miller, R. H., and Noble, M. D. (1983). A glial progenitor that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. *Nature* 303, 390–396.

Raff, M. C., Williams, B. P., and Miller, R. (1984). The in vitro differentiation of a bipotential glial progenitor cell. *EMBO J.* 3, 1857–1864.

Raff, M. C., Abney, E. R., and Fok-Seang, J. (1985). Reconstitution of a developmental clock in vitro: A critical role for astrocytes in the timing of oligodendrocyte differentiation. *Cell* 42, 61–69.

Raff, M. C., Lillien, L. E., Richardson, W. D., Burne, J. F., and Noble, M. D. (1988). Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature* 333, 562–565.

Ranscht, B., Clapshaw, P. A., Price, J., Noble, M., and Seifert, W. (1982). Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. *Proc. Nat. Acad. Sci. USA* 79, 2709–2713.

Richardson, W. D., Pringle, N., Mosely, M. J., Westermark, B., and Dubois-Dalcq, M. (1988). A role for platelet-derived growth factor in normal gliogenesis in the central nervous system. *Cell* 53, 309–319.

Saneto, R. P., and De Vellis, J. (1985). Characterization of cultured rat oligodendrocytes proliferating in a serum-free chemically defined medium. *Proc. Nat. Acad. Sci. USA* 82, 3509–3513.

Small, R. K., Riddle, P., and Noble, M. (1987). Evidence for migration of oligodendrocyte-type-2 astrocyte progenitor cells into the developing rat optic nerve. *Nature* 328, 155–157.

Szilard, L. (1959). On the nature of the aging process. *Proc. Natl. Acad. Sci. USA* 45, 30–45.

Temple, S., and Raff, M. C. (1985). Differentiation of a bipotential glial progenitor cell in single cell microculture. *Nature* 313, 223–225.

Temple, S., and Raff, M. C. (1986). Clonal analysis of oligodendrocyte development in culture: Evidence for a developmental clock that counts cell divisions. *Cell* 44, 773–779.

Weinberg, R. A. (1993). Tumor suppressor genes. *Neuron* 11, 191–196.

Wood, W. G., Bunch, C., Kelly, S., Cunn, Y., and Breckon, G. (1985). Control of hemoglobin switching by a developmental clock? *Nature* 313, 320–323.

Wren, D., Wolswijk, G., and Noble, M. (1992). In vitro analysis of the origin and maintenance of O-2A^{adult} progenitor cells. *J. Cell Biol.* 116, 167–176.

Zanjani, E. D., McGlave, P. B., Bhakthavathsalan, A., and Stamatoyannopoulos, G. (1979). Sheep fetal haematopoietic cells produce adult haemoglobin when transplanted in the adult animal. *Nature* 280, 495–496.

Table 3 Anti-PDGF antibodies block response of O-2A progenitors to type-1 astrocytes and PDGF, but not to FGF

	Radiolabelled O-2A progenitors (%)	
	No anti-PDGF	Plus anti-PDGF
ACM	63+4.2%	<1%
PDGF, 5 ng/ml	69+3.7%	<1%
FGF, 5 ng/ml	40+4.2%	44.5+2.5%

Cells were grown as in Table 1. Cultures received either the indicated mitogen, or mitogen plus 50 μg of rabbit anti-PDGF antiserum (purified Ig fraction, a kind gift of C. Heldin). When antibody was added, the ACM or non-conditioned medium with mitogen was preincubated with antibody for at least 1 h before addition to the cells. Cells were labelled with [^{3}H]-thymidine, immunolabelled, processed for autoradiography and scored as in Table 2.

the medium contained PDGF (data not shown). Thus, PDGF neither inhibited nor induced differentiation of O-2A progenitor cells into type-2 astrocytes, nor did it preclude differentiation of O-2A progenitor cells into oligodendrocytes. In these respects also, the effects of PDGF were identical to the effects of type-1 astrocytes.

To determine whether the effects of type-1 astrocytes were mediated by PDGF, we treated ACM, or medium containing PDGF or fibroblast growth factor (FGF) with affinity-purified anti-PDGF antibody. (As will be discussed elsewhere, FGF is also a mitogen for O-2A lineage cells, but does not mimic the effects of type-1 astrocytes on motility and differentiation.) These antibodies blocked the effects of ACM and PDGF, but did not block FGF-induced DNA synthesis in O-2A progenitors (Table 3), indicating that blocking was not due to toxic effects of the antibody.

Type-1 astrocytes also support the appropriately timed differentiation of embryonic O-2A progenitors grown *in vitro*[6]. As discussed in the accompanying paper[7], astrocyte-derived PDGF seems to play a key role in this effect and is by itself sufficient to promote the synchronous differentiation of clonally related and dividing progenitor cell families. Thus, in these respects also, PDGF completely mimics the effects of type-1 astrocytes.

The ability of a single molecule to replace type-1 astrocytes in modulating O-2A progenitor development *in vitro* suggests that these cells have a complex and constitutive behavioural phenotype, controlled by processes internal to the progenitors themselves. Progenitors stimulated to divide by an appropriate mitogen appear to be intrinsically migratory cells, with a bipolar morphology, which cease migration upon differentiating into multipolar oligodendrocytes; this differentiation seems to be controlled, at least in part, by internal clocks which may function by counting cell divisions. This programme does not, however, include astrocyte differentiation, which requires a separate inducing factor[10].

The observations that PDGF induced DNA synthesis at picomolar concentrations, the identical effects of PDGF and type-1 astrocytes on the division, differentiation and motility of O-2A progenitors and the ability of anti-PDGF antibodies to block the activity of astrocyte-conditioned medium all indicate that the astrocyte activity is a PDGF-like substance. In addition, messenger RNA for PDGF has been identified in purified astrocytes and these cells have been found to secrete PDGF *in vitro*[17] (W. Richardson, N. Pringle, M. Moseley, B. Westermark, & M. Dubois-Dalcq, manuscript submitted). Together, our studies indicate that PDGF may play an important role in gliogenesis in the CNS.

The ability of PDGF to promote division and migration of O-2A progenitor cells may be of particular interest in light of observations that PDGF can act as a chemotactic agent[11]. During embryogenesis, O-2A progenitors appear to populate the optic nerve by migrating from a germinal zone in or near the optic chiasm along the nerve towards the eye[5]. This directional migration could be due to movement along a gradient of a chemotactic substance, such as PDGF. In the adult animal, the ability of cells in a lesion site to secrete compounds which promote O-2A progenitor migration and division could be of value in the repair of demyelinating damage in the CNS. The controlled application of PDGF, or other chemotactic mitogens, might enhance these repair processes.

Received 25 February; accepted 29 April 1988.

1. Raff, M. C., Abney, E. R., Cohen, J., Lindsay, R. & Noble, M. *J. Neurosci.* 3, 1289–1300 (1983).
2. Noble, M. & Murray, K. *EMBO J.* 3, 2243–2247 (1984).
3. Raff, M. C., Miller, R. H. & Noble, M. *Nature* 303, 390–396 (1983).
4. Grench-Constant, C. & Raff, M. C. *Nature* 323, 335–338 (1986).
5. Small, R. K., Riddle, P. & Noble, M. *Nature* 328, 155–157 (1987).
6. Raff, M. C., Abney, E. R. & Fok-Seang, J. *Cell* 42, 61–69 (1985).
7. Raff, M. C., Lillien, L. E., Richardson, W. D., Burne, J. F. & Noble, M. D. *Nature* 333, 562–565.
8. Temple, S. & Raff, M. C. *Cell* 44, 773–779 (1986).
9. Mirsky, R. *et al. J. Cell Biol.* 84, 483–494 (1980).
10. Hughes, S. & Raff, M. C. *Development* 101, 157–167 (1987).
11. Ross, R., Raines, E. W. & Bowen-Pope, D. F. *Cell* 46, 155–169 (1986).
12. Stroobant, P. & Waterfield, M. D. *EMBO J.* 3, 2963–2967 (1984).
13. Eisenbarth, G. S., Walsh, F. S. & Nirnberg, M. *Proc. natn Acad. Sci. U.S.A.* 76, 4913–4917 (1979).
14. Ranscht, B., Clapshaw, P. A., Price, J., Noble, M. & Seifert *Proc. Natn. Acad. Sci. U.S.A.* 79, 2709–2713 (1982).
15. Bignami, A., Eng, L. F., Dahl, D. & Uyeda, C. T. *Brain Res.* 43, 429–435 (1972).
16. Pruss, R. M. *Nature* 280, 688–690 (1979).
17. Richardson, W. D., Pringle, N., Mosley, M. J., Westermark, B. & Dubois-Dalcq, M. *Cell* 53, 309–319 (1988).

Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture

Martin C. Raff*†, Laura E. Lillien*†, William D. Richardson†, Julia F. Burne*† & Mark D. Noble‡

* MRC Developmental Neurobiology Programme and † Biology Department, Medawar Building, University College London, London WC1E 6BT, UK
‡ Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT, UK

The various cell types in a multicellular animal differentiate on a predictable schedule but the mechanisms responsible for timing cell differentiation are largely unknown. We have studied a population of bipotential glial (O-2A) progenitor cells in the developing rat optic nerve[1] that gives rise to oligodendrocytes beginning at birth and to type-2 astrocytes[2] beginning in the second postnatal week[3]. Whereas, *in vivo*, these O-2A progenitor cells proliferate and give rise to postimitotic oligodendrocytes over several weeks[4,5], in serum-free (or low-serum) culture they stop dividing prematurely and differentiate into oligodendrocytes within two or three days[1,6,7]. The normal timing of oligodendrocyte development can be restored if embryonic optic-nerve cells are cultured in medium conditioned by type-1 astrocytes[8], the first glial cells to differentiate in the nerve[3]: in this case the progenitor cells continue to proliferate, the first oligodendrocytes appear on the equivalent of the day of birth, and new oligodendrocytes continue to develop over several weeks, just as *in vivo*[7]. Here we show that platelet-derived growth factor (PDGF) can replace type-1-astrocyte-conditioned medium in restoring the normal timing of oligodendrocyte differentiation *in vitro* and that anti-PDGF antibodies inhibit this property of the appropriately conditioned medium. We also show that PDGF is present in the developing optic nerve. These findings suggest that type-1-astrocyte-derived PDGF drives the clock that times oligodendrocyte development.

Table 1 Effect of PDGF on O-2A progenitor-cell proliferation and differentiation into oligodendrocytes *in vitro*

Age of optic nerve	PDGF (ng ml^{-1})	Days in culture	Number of O-2A progenitor cells	Number of oligodendrocytes
E17	0	2	8±5	15±7
E17	0	3	5±3	19±8
E17	2	2	51±8	0
E17	2	3	117±21	0
E17	2	4	214±38	4±2
E18	0	2	8±3	55±7
E18	2	2	135±17	0
E18	2	3	317±45	10±4
E19	0	1	14±2	70±4
E19	2	1	120±9	0
E19	2	2	199±28	2±1
E18	2	2	148±13	0
E18	2	3	287±23	12±6
E18	10	2	133±11	0
E18	10	3	278±37	8±4

Optic nerves from embryonic S/D rats were dissociated into single cells and cultured on poly-D-lysine (PDL)-coated glass coverslips (about 30,000 cells per coverslip) in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose, insulin, transferrin, bovine serum albumin, progesterone, putrescine, thyroxine, tri-idodothyronine and 0.5% FCS as described[7]. Purified human PDGF (R and D Systems, Inc.) was added at the start of the culture. After 1–4 days, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (*p*H 7.5) for 5 min at room temperature and stained successively with monoclonal anti-galactocerebroside (GC) antibody[19] (ascites fluid diluted 1:1000), fluorescein-coupled goat anti-mouse IgG3 (G anti-IgG3-Fl, Nordic, diluted 1:100), A2B5 monoclonal antibody[20] (ascites fluid diluted 1:100) and finally rhodamine-coupled goat anti-mouse Ig (G anti-MIg-Rd, Cappel, diluted 1:100); the cells were then post-fixed in acid-alcohol, mounted in glycerol and examined in a Zeiss Universal fluorescence microscope, as described[7]. O-2A progenitor cells were identified by their antigenic phenotype (A2B5$^+$, GC$^-$)[1,15] and characteristic process-bearing morphology[15], while oligodendrocytes were identified as GC$^+$ process-bearing cells[11]. The total numbers of these cells were counted on each coverslip and the results are expressed as means ± s.d. of at least three experiments. The concentration of PDGF required for half-maximal stimulation of O-2A progenitor-cell proliferation was ~0.5 ng ml^{-1} (ref. 9).

We were led to study the effect of PDGF on the timing of oligodendrocyte development *in vitro* by recent evidence that PDGF is a potent mitogen for O-2A progenitor cells in culture[9,10] and that cultured type-1 astrocytes stimulate O-2A progenitor-cell proliferation[8] by secreting PDGF[9]. In the present study, optic nerve cells from embryonic day 17 (E17) Sprague–Dawley (S/D) rats were cultured in medium containing 0.5% fetal calf serum (FCS). As previously reported[7], within two days most of the O-2A progenitor cells in such cultures stopped dividing and differentiated into oligodendrocytes, which were identified by the binding of antibody against galactocerebroside (GC)[11] (Table 1). When human PDGF (R and D Systems, Inc., Minneapolis) was added, however, the O-2A progenitor cells continued to proliferate, doubling in number approximately every day, and the first oligodendrocytes developed after four days, equivalent to the time of birth (Table 1). The same result was obtained if the cells were cultured in type-1-astrocyte-conditioned medium (ACM), as reported previously[7] (data not shown). When E18 or E19 optic nerve cells were cultured in PDGF, the first oligodendrocytes developed after three and two days, respectively, again equivalent to the day of birth (Table 1). The same results were obtained with a fivefold higher concentration of PDGF (Table 1), with human PDGF obtained from Raines and Ross[12], and with porcine PDGF obtained either from R and D Systems or from Stroobant and Waterfield[13] (data not shown).

These results indicate that PDGF can mimic the effects of ACM in restoring in culture the normal timing of oligodendrocyte development observed *in vivo*, raising the possibility that PDGF is the factor in ACM responsible for this activity. To test this possibility, E17 optic nerve cells were cultured in ACM together with an IgG fraction of a goat anti-PDGF antiserum. As shown in Table 2, these antibodies completely blocked the ability of ACM both to stimulate O-2A progenitor-cell proliferation (as reported previously[9]) and to restore the normal *in vivo* timing of oligodendrocyte development in culture (Table 2). The same result was obtained using an IgG fraction of a rabbit antiserum[14] obtained from C.-H. Heldin (data not shown). IgG fractions of goat and rabbit antisera against mouse immunoglobulin, used at the same or tenfold higher concentration, had no such effect (data not shown). The addition of exogenous PDGF together with the anti-PDGF antibodies completely overcame the inhibitory activity of the antibodies (Table 2).

Table 2 Effect of anti-PDGF antibodies on the ability of conditioned medium (ACM or ONCM) to restore the *in vivo* timing of oligodendrocyte development in cultures of E17 optic nerve cells

Conditioned medium	Anti-PDGF antibodies (90 μg ml^{-1})	PDGF (ng ml^{-1})	Number of O-2A progenitor cells	Number of oligodendrocytes
none	−	0	12±5	25±11
none	−	2	71±9	0
ACM	−	0	75±13	0
ACM	+	0	15±7	24±8
ACM	+	15	82±18	0
ONCM	−	0	64±8	0
ONCM	+	0	8±2	31±5

Cells from E17 optic nerves were prepared, cultured for 2 days and stained as in Table 1. Purified type-1 astrocytes from newborn rat cerebral cortex were prepared as described[21]; after growing for several weeks in DMEM supplemented with 10% FCS, the astrocytes were grown in the defined medium (with 0.5% FCS) described in Table 1 for 2 days and the medium was collected as ACM. Newborn optic nerve cells (5×10^5 in 2 ml in PDL-coated 3.5 mm Nunc tissue-culture dishes) were cultured as described[1]; after 1 day in DMEM containing 10% FCS, the cultures were switched to defined medium containing 0.5% FCS, which was collected after 1 day as ONCM. The conditioned media were tested on E17 optic-nerve cultures to find the highest dilution that would still restore the normal timing of oligodendrocyte differentiation, and this concentration (which varied from undiluted to diluted 1:10) was used in these experiments. The medium was changed after 24 h, and fresh conditioned medium, anti-PDGF antibody, and human PDGF (R and D Systems, Inc.) were added. The goat anti-human PDGF antibodies (an IgG fraction prepared by ion-exchange chromatography) were purchased from Collaborative Research Inc. (Bedford, Massachusetts); 50 μg ml^{-1} of the antibody completely neutralized the mitogenic activity of 5 ng ml^{-1} of human PDGF for O-2A progenitor cells (data not shown), and for NIH 3T3 cells (according to the Collaborative Research specification sheet). The results are expressed as means ± s.d. of three separate experiments, except for the results with ONCM where they are triplicates of a single experiment.

The ACM used in the present and previous studies[7–9] was derived from cultures of type-1 astrocytes purified from neonatal-rat cerebral cortex. To determine whether type-1 astrocytes in optic nerve cell cultures also secrete PDGF, we cultured E17 optic nerve cells in medium conditioned over a high density culture of newborn optic nerve cells; almost 60% of newborn optic nerve cells are type-1 astrocytes[3]. As shown in Table 2, such optic nerve conditioned medium (ONCM) kept O-2A progenitor cells dividing and prevented these cells from prematurely differentiating into oligodendrocytes; this activity was

completely inhibited by anti-PDGF antibodies. We have previously provided evidence, however, that the endogenous type-1 astrocytes in E17 optic nerve cultures are too few in number and/or are unable to recover quickly enough from the dissociation procedure to produce sufficient mitogen to keep the progenitor cells dividing and to prevent their premature differentiation[7].

To determine whether PDGF is made in the developing optic nerve, we tested an extract of three-week-old rat optic nerve for its ability to stimulate O-2A progenitor cells in culture to incorporate bromodeoxyuridine (BrdU) into DNA before and after the extract was treated with anti-PDGF antibodies. As shown in Table 3, the extract stimulated progenitor cells to incorporate

Table 3 Stimulation of BrdU incorporation in O-2A progenitor cells by optic nerve extract in culture: the effect of anti-PDGF antibodies

Additives	% O-2A progenitor cells labelled with BrdU
None	3±1
PDGF (1ng ml^{-1})	64±4
Optic nerve extract	50±2
Optic nerve extract treated with anti-PDGF antibodies	14±6

Cells from newborn optic nerves were cultured as in Table 1, except that they were maintained without FCS at 5,000 cells per culture. Bromodeoxyuridine (BrdU, 10 μM; Boehringer) was added after 11.5 h, and the cultures were fixed after 48 h and stained with A2B5 antibody, followed by G anti-MIg-F1 (Cappel, 1:100); they were then treated successively with 2 N HCl (to denature the nuclear DNA[22]), 0.1 M $Na_2B_4O_7$, *p*H 8.5 (each for 10 min at room temperature), monoclonal anti-BrdU antibody[23] (culture supernatant diluted 1:5) and finally with G ani-MIgRd. Optic-nerve extract was prepared from three week-old rats as described[18] and used at 220 μg total protein ml^{-1}. For treatment with anti-PDGF antibodies, the extract (264 μg total protein) was incubated with the IgG fraction of goat anti-human PDGF antiserum (135 μg in 0.7 ml of DMEM; Collaborative Research) for 4 h at 4 °C with continuous rotation; protein A-Sepharose (50 μl of swollen gel, Pharmacia) was added and the mixture was incubated for a further 12 h at 4 °C with continuous rotation and then centrifuged for 1 min in an MSE Micro Centaur. Incubating the extract in normal goat serum and then protein A-Sepharose had no effect on the extract's activity; the extract used in the experiment shown was treated in this way. Anti-PDGF antibodies inhibited the activity of the extract to the same extent when they were added directly (85 μg ml^{-1}) to the cultures (data not shown). The results are expressed as means ± s.e.m. of triplicate cultures.

BrdU, and more than 70% of this mitogenic activity was removed by anti-PDGF antibodies. While this suggests that the major mitogen for O-2A progenitor cells in the optic nerve at this age is PDGF, the finding that not all of the mitogenic activity in the extract was removed by the antibodies, or neutralized when progenitor cells were cultured with the extract in the presence of an excess of anti-PDGF antibody (data not shown), suggests that another mitogen(s) is also present.

Our present and previous results provide compelling evidence that PDGF, secreted by type-1 astrocytes, regulates both the proliferation and the timing of differentiation of O-2A progenitor cells *in vitro*. To summarize the evidence: (1) PDGF stimulates the proliferation of O-2A progenitor cells and prevents them from differentiating prematurely into oligodendrocytes in culture (refs 9, 10, and this study); (2) cultures of type-1 astrocytes purified from cerebral cortex make both PDGF and messenger RNA encoding PDGF A chains[9]; (3) when ACM is fractionated by gel filtration, the mitogenic activity for O-2A progenitor cells is found in the same fractions as radiolabelled PDGF[9]; (4) anti-PDGF antibodies inhibit the ability of ACM and ONCM to stimulate O-2A progenitor cell proliferation *in vitro* (ref. 9, 10 and this study) and to restore the normal *in vivo* timing to oligodendrocyte differentiation (this study).

Table 4 Clonal analysis of oligodendrocyte differentiation in optic nerve cell cultures from 7-day-old rats stimulated by PDGF

Clones	Number of cell divisions: 0	1	2	3	4
a,b,c	1P	2ol			
d,e,f	1P	2P	4ol		
g	1P	2P	4P	8ol	
h	1P	2P	3P,1M	6ol	
i	1P	2P	4P	8P	16ol
j	1P	2P	4P	5P,1ol,1M	11ol

Cells from post-natal day 7 (P7) S/D rats were prepared and studied by time-lapse microcinematography as described[16], except that the cells were maintained in human PDGF (10 ng ml^{-1}) instead of ACM. Proliferation and differentiation of cells in microscopic fields containing 2 to 4 O-2A progenitor cells were followed for one week. The O-2A progenitor cells were identified by their characteristic bipolar morphology[15,16] and migratory behaviour[16], while oligodendrocytes were identified by their multipolar morphology[10,15,16] and lack of motility[16]. As shown in the table, oligodendrocyte differentiation occurred after one to four cell divisions in the ten clones studied, although in every experiment there were still dividing O-2A progenitor cells (belonging to other clones) present in the field at the end of filming. P, O-2A progenitor cells; ol, oligodendrocytes; M, cells that migrated out of the microscopic field and could no longer be followed.

Previous studies have suggested that ACM regulates the timing of oligodendrocyte development *in vitro* by keeping O-2A progenitor cells dividing until an intrinsic clock in the progenitor cell initiates the process that leads to oligodendrocyte differentiation[7,15]. The results of these studies were consistent with the possibility that the clock operates by setting a maximum number of divisions a progenitor cell and its progeny can undergo before differentiating. To test this possibility further we cultured optic nerve cells from seven-day-old rats in the presence of PDGF and followed the fate of individual O-2A progenitor cells and their progeny by time-lapse microcinematography. As described previously, O-2A progenitor cells and oligodendrocytes could be easily recognized as motile bipolar cells and immotile multipolar cells, respectively[16]. In nine of the ten clones studied, all of the descendants of a single progenitor cell differentiated together into non-dividing oligodendrocytes after the same number of cell divisions (Table 4); in the other clone (clone j in Table 4), the progenitor cells differentiated within one cell division of one another. These findings are consistent with previous single-cell experiments and the 'mitotic clock' hypothesis[15] but do not exclude other timing mechanisms.

Whatever the timing mechanism, it is clear that oligodendrocyte differentiation is associated with withdrawal from the cell cycle[4,5,8]. The relationship between the two processes, however, is uncertain. The clock in the progenitor cell might primarily control the onset of oligodendrocyte differentiation, with the cessation of proliferation following as a consequence. Alternatively, the clock might primarily control the onset of unresponsiveness to PDGF, with oligodendrocyte differentiation following as a consequence of withdrawal from the cell cycle. We favour the second possibility as it would most simply explain why O-2A progenitor cells differentiate prematurely when deprived of PDGF (see Table 2).

Whereas oligodendrocyte differentiation seems to be the constitutive pathway of O-2A progenitor cell development, which is automatically triggered when a progenitor cell is deprived of signals from other cells[17] or when the instrinsic timer reaches the appropriate point[7,15], type-2 astrocyte differentiation seems to be induced by a protein signal that greatly increases in concentration in the optic nerve after the first postnatal week[18]. While the mechanisms that control the timing and direction of O-2A progenitor cell differentiation *in vitro* are beginning to emerge, it remains to show that the same mechanisms operate *in vivo*.

We thank E. Raines, R. Ross, P. Stroobant and M. Waterfield for gifts of PDGF, C.-H. Heldin for a gift of anti-PDGF antibodies, P. Riddle for help with the time-lapse microcinematography and A. Mudge for helpful comments on the manuscript. L.E.L. was supported by a Fellowship from the National Multiple Sclerosis Society of the United States and W.D.R. by an MRC Project Grant.

Received 4 April; accepted 29 April 1988.

1. Raff, M. C., Miller, R. H. & Noble, M. *Nature* 303, 390–396 (1983).
2. Raff, M. C., Abney, E. R., Cohen, J., Lindsay, R. & Noble, M. *J. Neurosci.* 3, 1289–1300 (1983).
3. Miller, R. H., David, S., Patel, R., Abney, E. R. & Raff, M. C. *Devl Biol.* 111, 35–41 (1985).
4. Skoff, R. P., Price, D. L. & Stocks, A. *J. comp. Neurol.* 169, 291–312 (1976).
5. Skoff, R., Price, D. & Stocks, A. *J. comp. Neurol.* 169, 313–333 (1976).
6. Raff, M. C., Williams, B. P. & Miller, R. H. *EMBO J.* 3, 1857–1864 (1984).
7. Raff, M. C., Abney, E. R. & Fok-Seang, J. *Cell* 42, 61–69 (1985).
8. Noble, M. & Murray, K. *EMBO J.* 3, 2243–2247 (1984).
9. Richardson, W. D., Pringle, N., Mosley, M., Westermark, B. & Dubois-Dalcq, M. *Cell* 53, 309–319 (1988).
10. Noble, M., Murray, K., Stroobant, P., Waterfield, M. D & Riddle, P. *Nature* 333, 560–562 (1988).
11. Raff, M. C. *et al. Nature* 274, 813–816 (1978).
12. Raines, B. & Ross, R. *J. biol. chem.* 257, 5154–5160 (1982).
13. Stroobant, P. & Waterfield, M. *EMBO J.* 3, 2963–2967 (1984).
14. Heldin, C.-H., Westermark, B. & Wasteson, A. *Expl Cell Res.* 136, 255–261 (1981).
15. Temple, S. & Raff, M. C. *Cell* 44, 773–779 (1986).
16. Small, R. K., Riddle, P. & Noble, M. *Nature* 329, 155–157 (1987).
17. Temple, S. & Raff, M. C. *Nature* 313, 223–225 (1985).
18. Hughes, S. & Raff, M. C. *Development* 101, 157–167 (1987).
19. Ranscht, B., Clapshaw, P. A., Price, J., Noble, M. & Seifert, W. *Proc. natn. Acad. Sci. U.S.A.* 79, 2709–2713 (1982).
20. Eisenbarth, G. S., Walsh, F. S. & Nirenberg, M. *Proc. natn. Acad. Sci. U.S.A.* 76, 4913–4917 (1979).
21. Janzer, R. C. & Raff, M. C. *Nature* 325, 253–257 (1987).
22. Yong, V. W. & Kim, S. U. *J. Neurosci. Meth.* 21, 9–16 (1987).
23. Magaud, J.-P. and Masson, D. Y. *J. immunol. Meth.* (in the press).

The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria

Periasamy Selvaraj, Wendall F. Rosse*, Robert Silber† & Timothy A. Springer

Laboratory of Membrane Immunochemistry, Dana Farber Cancer Institute, Pathology Department, Harvard Medical School, Boston, Massachusetts 02115, USA
* Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710, USA
† Department of Medicine, New York University Medical Center, New York 10016, USA

Fc receptors on phagocytic cells in the blood mediate binding and clearance of immune complexes, phagocytosis of antibody-opsonized microorganisms, and potently trigger effector functions, including superoxide anion production and antibody-dependent cellular cytotoxicity. The Fc receptor type III (FcγR III, CD16), present in 135,000 sites per cell 1 on neutrophils and accounting for most of FcR in blood, unexpectedly has a phosphatidylinositol glycan (PIG) membrane anchor. Deficiency of FcγR III is observed in paroxysmal nocturnal haemoglobinuria (PNH), an acquired abnormality of haematopoietic cells[2] affecting PIG tail biosynthesis or attachment[3], and is probably responsible for circulating immune complexes[4] and susceptibility to bacterial infections associated with this disease[5]. Although a growing number of eukaryotic cell-surface proteins with PIG-tails are being described[6,7], none has thus far been implicated in receptor-mediated endocytosis or in triggering of cell-mediated killing. Our findings on the FcγR III raise the question of how a PIG-tailed protein important in immune complex clearance *in vivo*[8,9] and in antibody-dependent killing[10] mediates ligand internalization and cytotoxicity. Together with our results, previous functional studies on FcγR III and FcγR II[11,12] suggest that these two receptors may cooperate and that the type of membrane anchor is an important mechanism whereby the functional capacity of surface receptors can be regulated.

Three different types of FcγR have been distinguished in humans using monoclonal antibodies[13] (mAb). FcγR III (CD16) of relative molecular mass (M_r) 50–70,000 (50–70K) is found on neutrophils, large granular lymphocytes, and macrophages, but not on monocytes. FcγR III was first identified with a mAb (3G8) that blocked immune complex binding to neutrophils[1] and subsequently with other mAb of the CD16 cluster[14]. FcγR II (CDw32) is a 40K receptor on neutrophils, monocytes, eosinophils, platelets and B cells[13]. FcγR I is a 72K protein and is found on monocytes[13]. FcγR III and FcγR II have low affinity for monomeric IgG and thus preferentially bind immune complexes by multiple receptor–ligand interactions, whereas FcγR I is sufficiently high affinity to bind monomeric IgG.

Our first evidence that FcγR III is anchored by PIG came from studies on leukocytes from patients with paroxysmal nocturnal haemoglobinuria (PNH). PNH is an acquired defect of haematopoietic precursor cells in either the biosynthesis or the attachment of the PIG tail and may affect clonal progeny in the erythroid, monocytic, granulocytic, and thrombocytic lineages[2,15,16]. Previous studies on erythrocytes and leukocytes from PNH patients have demonstrated a selective deficiency of PIG-tailed proteins, including decay accelerating factor (DAF), acetylcholinesterase, alkaline phosphatase and the PIG-anchored form of lymphocyte function-associated antigen 3 (LFA-3), (refs 3, 6 and 7). The deficiency of DAF accounts for susceptibility of erythrocytes to complement-mediated lysis in PNH. However none of these previously identified deficiencies can explain the occurrence of circulating immune complexes[4] and the 20% and 50% of mortalities caused by bacterial infections and thrombosis respectively[5].

Quantitation of FcγR III expression using immunofluorescence flow cytometry show that it is markedly deficient on PNH neutrophils (Fig. 1*a*). This deficiency was found in all six patients studied (D.E., S.B., J.M., J.E., B.I., C.G.) and results with five different CD16 (FcγR III) mAb were identical. Some patients such as J.E. (Fig. 1*a*, curve 3) showed normal as well as deficient granulocyte clones. Patients showed consistent variation in the extent of deficiency in the abnormal clone. The amount of FcγR III expression on affected cells ranged from 2% (patient D.E.) to 19% (patient J.E.) averaging 7% of normal, perhaps reflecting the degree of penetrance of the acquired defect in PNH. In all cases, deficiency of FcγRIII paralleled deficiency of DAF. In contrast, deficient neutrophils expressed normal levels of HLA-A,B, LFA-1, Mac-1 and FcγR II (CDw32) (Fig. 1*a*). PNH monocytes showed normal expression of FcγR I and II, although they were deficient in DAF (Fig. 1*b*). These results suggested that the neutrophil FcγR III has a PIG tail, whereas the FcγR I and FcγR II do not.

PIG-anchored proteins can be specifically cleaved from cell surfaces with phosphatidylinositol-specific phospholipase C[3,6,7] (PIPLC). We therefore investigated the susceptibility of Fc receptors to PIPLC (Table 1). PIPLC released 75-84% of the cell surface FcγR III and DAF from healthy granulocytes, while FcγR II, HLA-A,B and LFA-1 were unaffected. On monocytes, PIPLC released 84% of DAF whereas it had no effect on FcγR II, FcγR I, HLA-A,B and LFA-1. Results with PIPLC prepared from *S. aureus* and *B. thuringiensis* were identical and show that FcγR III on neutrophils, but not FcγR II on the same cells or FcγR I and II on monocytes, have PIG anchors. Lack of a PIG anchor on FcγR II is consistent with the prediction (from cDNA sequence[17]) that it possesses a transmembrane domain and a 76 residue hydrophilic cytoplasmic tail.

Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain

MARTA C. NUNES[1], NEETA SINGH ROY[1], H. MICHAEL KEYOUNG[1], ROBERT R. GOODMAN[2], GUY MCKHANN II[2], LI JIANG[3], JIAN KANG[3], MAIKEN NEDERGAARD[3] & STEVEN A. GOLDMAN[1]

[1]*Department of Neurology and Neuroscience, Cornell University Medical College, New York, New York, USA*
[2]*Department of Neurosurgery, Columbia University College of Physicians and Surgeons, New York, New York, USA*
[3]*Department of Anatomy and Cell Biology, New York Medical College, Valhalla, New York, USA*
M.C.N. and N.S.R. contributed equally to this work.
Correspondence should be addressed to S.A.G.; e-mail: sgoldm@mail.med.cornell.edu

Published online 10 March 2003; doi:10.1038/nm837

The subcortical white matter of the adult human brain harbors a pool of glial progenitor cells. These cells can be isolated by fluorescence-activated cell sorting (FACS) after either transfection with green fluorescent protein (GFP) under the control of the *CNP2* promoter, or A2B5-targeted immunotagging. Although these cells give rise largely to oligodendrocytes, in low-density culture we observed that some also generated neurons. We thus asked whether these nominally glial progenitors might include multipotential progenitor cells capable of neurogenesis. We found that adult human white-matter progenitor cells (WMPCs) could be passaged as neurospheres *in vitro* and that these cells generated functionally competent neurons and glia both *in vitro* and after xenograft to the fetal rat brain. WMPCs were able to produce neurons after their initial isolation and did not require *in vitro* expansion or reprogramming to do so. These experiments indicate that an abundant pool of mitotically competent neurogenic progenitor cells resides in the adult human white matter.

The adult human subcortical white matter harbors a population of mitotically competent glial progenitors that comprise as many as 3% of its cells[1,2]. These cells may be extracted from brain tissue using FACS after transfection with GFP-encoding plasmids driven by the promoter for *CNP*, an early oligodendrocytic transcript[2,3]. The cells express the immature neural ganglioside recognized by monoclonal antibody A2B5 but do not express more mature markers of glial lineage. We previously noted that when grown at high density, pCNP2:hGFP$^+$ progenitors gave rise to glia, largely oligodendrocytes. Nonetheless, in low-density culture after high-purity FACS, pCNP2:hGFP$^+$ cells often generated βIII-tubulin$^+$ neurons[2]. Because neurogenesis was never observed from pCNP2:hGFP$^+$ cells in higher-density or unsorted cultures, we postulated that the restriction of these progenitor cells to the oligodendroglial phenotype might be an effect of environmental cues rather than a function of autonomous commitment. Once isolated into high-purity, low-density culture, and therefore removed from any paracrine or autocrine influences, human subcortical pCNP2:hGFP$^+$ cells were able to generate neurons as well as glia[2]. It was subsequently reported[4] that glial progenitors from the postnatal rat optic nerve could also generate neurons after serum- or bone morphogenetic protein–induced phenotypic instruction and basic fibroblast growth factor (bFGF)–stimulated expansion. Similar work showed that progenitor cells of the adult rat forebrain parenchyma could also generate neurons after prolonged *in vitro* expansion in bFGF[8]. Taken together, these findings indicated that glial progenitor cells might retain substantial phenotypic plasticity.

We asked whether some fraction of the nominally glial progenitors of the adult human subcortical white matter might actually be parenchymal neural stem cells. Specifically, we asked whether single, sorted WMPCs could generate multiple neural phenotypes, and if so, whether they were capable of expansion and self-renewal. In addition, we investigated whether this process requires de-differentiative reprogramming to an intermediate phenotype, or whether simply removing these cells from their local environment and mitotically expanding them in bFGF might suffice to permit these cells to act as multipotential progenitors. In doing so, we tested the hypothesis that the phenotypic plasticity of adult WMPCs might be tonically restricted by the adult parenchymal environment, rather than irreversibly lost with development.

WMPCs were isolated by *CNP*- and A2B5-based sorting

White matter was dissected from surgical samples taken at the time of temporal lobectomy for epilepsy, aneurysm, and post-traumatic decompression ($n = 21$). The tissues were dissected free of adjacent cortex and ventricular epithelium, and enzymatically dissociated to single-cell suspension as described[2]. The dissociates were plated onto laminin (100 μg/ml) in DMEM/F12/N1 supplemented with bFGF (20 ng/ml), NT3 (2 ng/ml) and platelet-derived growth factor (PDGF)-AA (20 ng/ml). To identify oligodendrocyte progenitors, the dissociates were trans-

fected with pCNP2:hGFP, the transcription of which results in GFP expression by oligodendrocyte progenitor cells[2].

To avoid both the temporal lag between transfection and GFP expression and the inefficiency of plasmid transfection, cultures were also sorted on the basis of A2B5 surface immunoreactivity, which can serve as a surrogate marker for pCNP2:hGFP$^+$ WMPCs *in vitro*[2]. Immunostaining showed that 84 ± 8.3% of pCNP2:GFP$^+$ cells expressed A2B5 (ref. 2). GFP-based FACS gated 0.49 ± 0.15% of all white-matter cells as pCNP2:hGFP$^+$ (mean ± s.e.m.; $n = 3$ patients; Fig. 1*a*). Matched cultures transfected with pCMV:GFP had a net transfection efficiency of 13.1%. Thus, the predicted incidence of pCNP2:hGFP$^+$ cells in the white matter was 3.7% (= 1 + 0.131 × 0.49), consistent with our prior estimates of the incidence of this phenotype[2]. From the same samples, A2B5-based FACS gated an average of 3.1 ± 0.7% ($n = 3$) of the white-matter cell population (Fig. 1*b*). The greater than six-fold increase in net yield when A2B5 was used (3.1% versus 0.49%) reflected the higher efficiency of A2B5 immunodetection relative to pCNP2:hGFP plasmid transfection. On this basis, we used immunomagnetic sorting (IMS) to select A2B5$^+$ cells from adult white-matter dissociates. By IMS, the incidence of A2B5-sorted cells in white matter dissociates was 3.6 ± 0.3% ($n = 21$) with a median of 3.1%. This improved yield was accomplished with no appreciable loss of cell-type specificity, in that the A2B5$^+$ cells overlapped entirely with the sort profiles of pCNP2:hGFP$^+$ cells and each isolate generated O4$^+$ oligodendrocytes with similar efficiency (Fig. 1*c–f*). Thus, A2B5-based FACS and IMS identified WMPCs homologous to those recognized by pCNP2:GFP-based FACS, while permitting higher-yield isolation of these cells.

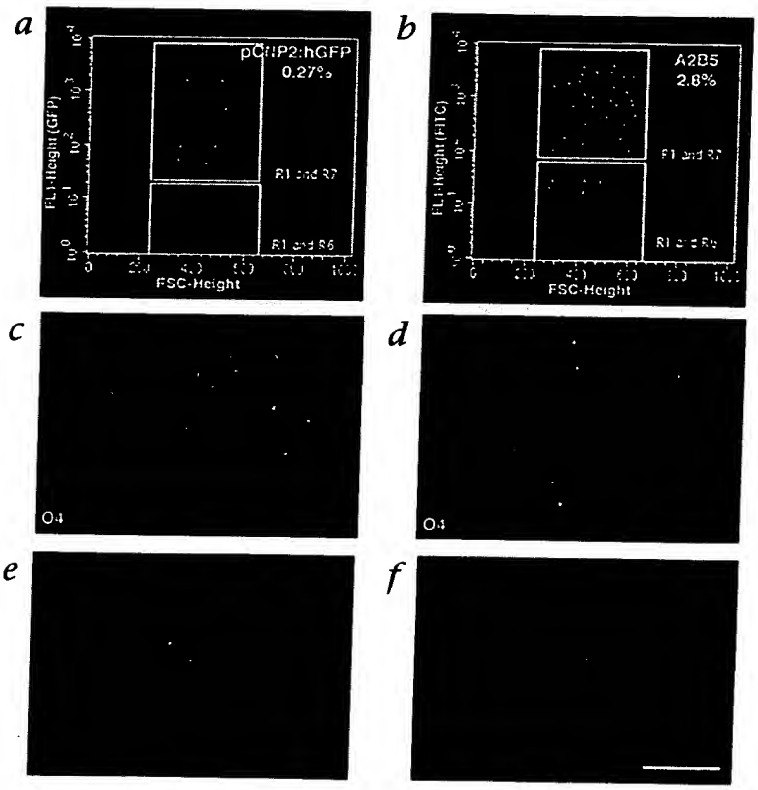

Fig. 1 A2B5-based FACS selects oligodendrocyte progenitor cells. ***a*** and ***b***, FACS graphs showing the extraction of pCNP2:hGFP$^+$ (*a*) and A2B5$^+$ (*b*) WMPCs from an adult human white-matter dissociate. Forward scatter (FCS), a measure of cell size, is plotted against fluorescence intensity (Fl-1). When pCNP2:hGFP- and A2B5-based sorts were directly compared, their plots showed overlapping profiles, but A2B5$^+$ cells were >6-fold more abundant than their pCNP2:hGFP$^+$ counterparts, reflecting the higher efficiency of A2B5 surface tagging. ***c–f***, Progenitors sorted by pCNP2:hGFP (*c* and *e*) and A2B5 (*d* and *f*) gave rise to O4$^+$ oligodendrocytes. A2B5-based surface antigen sorting may thus be used as a higher-yield alternative to pCNP2:hGFP transfection–based FACS for isolating WMPCs. Scale bar, 24 μm.

Adult WMPCs gave rise to multipotent neurospheres

To assess the expansion capacity of pCNP2:hGFP- and A2B5-sorted cells, we propagated sorted isolates of each in suspension[6–8]. The cells were distributed into 24-well plates at 50,000 cells per 0.5 ml in serum-free media (SFM) supplemented with bFGF (20 ng/ml), NT3 (2 ng/ml) and PDGF-AA (20 ng/ml), a combination that permits the expansion of human WMPCs[2]. Seven days later, the cells were switched to SFM with bFGF alone (20 ng/ml)[8]. Over the next 10 d, neurospheres—spherical masses of cells that expand from single parental progenitors—arose in these cultures, such that by 3 weeks after sorting, there were 84.8 ± 9.0 spheres/well (n = 4 patients). These neurospheres were typically >150 μm in diameter and included 46.5 ± 8.2 cells/sphere (Fig. 2*a* and *b*). Thus, single WMPCs of the adult human brain were capable of generating neurospheres.

To establish the lineage potential of single adult human WMPCs, we dissociated the resultant primary neurospheres and passaged them into new wells. Alternatively, some were plated onto substrate to permit their differentiation. Immunostaining showed that both pCNP2:hGFP$^+$ and A2B5$^+$ progenitor–derived spheres gave rise to all major neural phenotypes (Fig. 2*d* and *e*). Among those cells passaged from primary spheres, secondary spheres were observed to arise within two weeks after passage. After expansion, these secondary spheres were similarly plated on substrate, raised for one to two weeks and fixed. Immunolabeling confirmed that virtually all secondary spheres generated both neurons and glia together (Fig. 2*c* and *e*). In addition, when the mitotic marker BrdU was added to A2B5-sorted cells, BrdU-incorporating neurons, oligodendrocytes and astrocytes all emerged from the spheres generated (Fig. 2*f–i*). The persistence of mitotic neurogenesis and gliogenesis by single spheres indicated that they contained cycling multipotential cells. The secondary spheres were probably of clonal origin, given the low plating density of the single cells from which each was derived and the fact that the sphere-forming cells originated from primary spheres that had themselves expanded from single-cell dissociates. These data indicate that single progenitor cells of the adult human white matter are both clonogenic and multipotent.

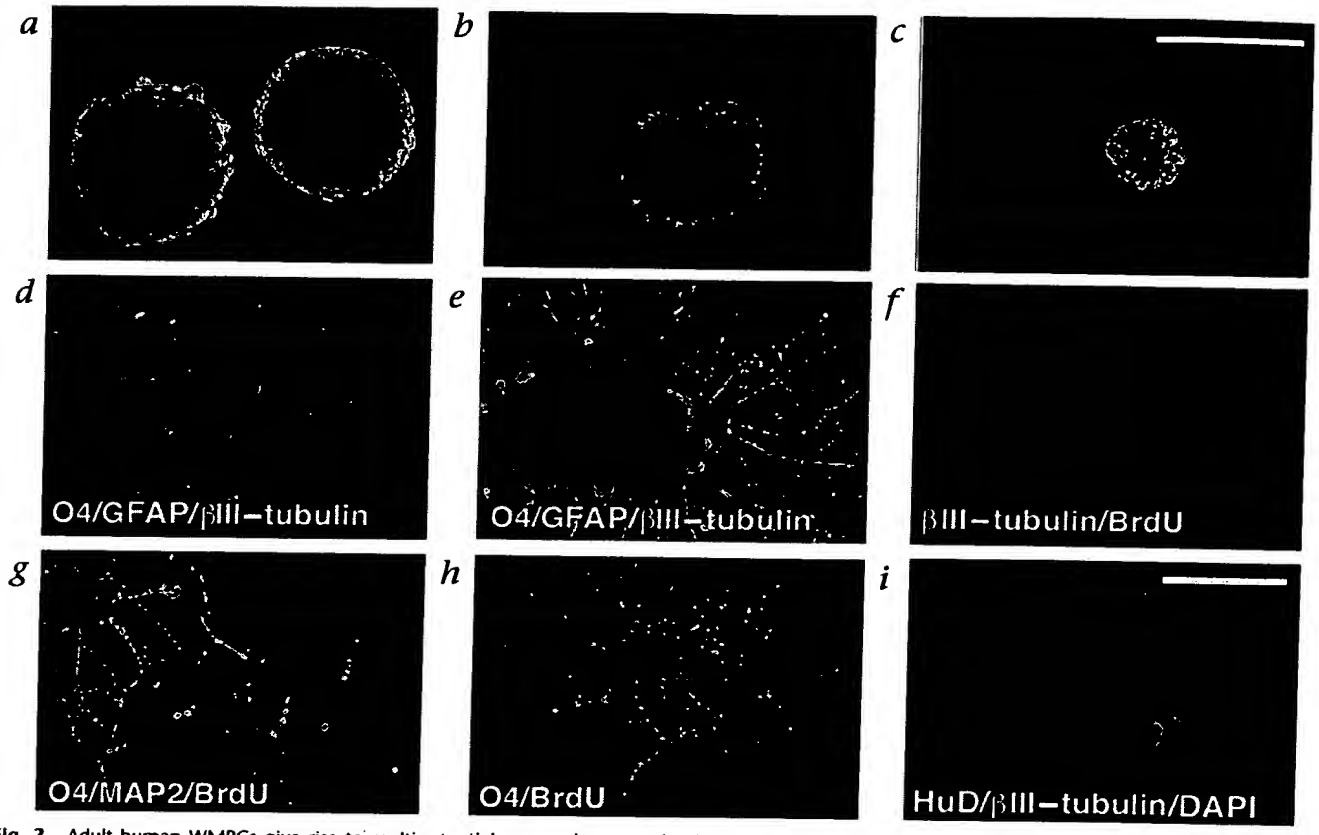

Fig. 2 Adult human WMPCs give rise to multipotential neurospheres. ***a***, First-passage spheres generated from A2B5-sorted cells 2 weeks after sorting. ***b***, First-passage spheres arising from pCNP2:hGFP-sorted cells, at 2 weeks. ***c***, Second-passage sphere derived from an A2B5-sorted sample, at 3 weeks. ***d***, Once plated onto substrate, the primary spheres differentiated into βIII-tubulin$^+$ neurons (red), GFAP$^+$ astrocytes (blue) and O4$^+$ oligodendrocytes (green). ***e***, Neurons (red), astrocytes (blue) and oligodendrocytes (green) arose similarly from spheres derived from pCNP2:GFP-sorted WMPCs. ***f–h***, BrdU incorporation (blue) showed that new neurons (*f*, βIII-tubulin (red); *g*, MAP2 (red)) and oligodendrocytes (*h*, O4 (green)) were generated *in vitro*. ***i***, βIII-tubulin$^+$ neurons (green) co-expressed neuronal Hu protein[40,41] (red), yielding double labeling (yellow). Nuclei were counterstained with DAPI (blue). Scale bars, 100 μm (*a–e*) or 40 μm (*f–i*).

Single WMPCs remained multipotential with passage

The serial propagability of sorted WMPCs from neurospheres in low-density dissociates suggested the clonal derivation of each individual sphere[9–11]. To further validate the clonal origin of neurons and glia arising within single spheres, we used lentiviral GFP to genetically tag and follow single WMPCs. A2B5$^+$ cells were tagged, 2–5 d after sorting, with a lentivirus expressing GFP under cytomegalovirus (CMV) promoter control[12–14]. At 10 PFU/cell, 23% of the cells expressed GFP by one week after sorting, yielding a mixture of GFP$^-$, GFP$^+$ and mixed spheres in the resultant cultures (Fig. 3*a–b*). These primary spheres were triturated two to four weeks later to single-cell suspensions and passaged into bFGF at ~3,000 cells/well. Under these conditions, 40.8 ± 12.9 secondary spheres/well were generated, indicating a clonogenic cell incidence of 1.3% (n = 5). Of these secondary spheres, 47.2 ± 10.8% contained only GFP$^+$ cells (Fig. 3*c–d*) whereas 30.9 ± 6.9% harbored no GFP$^+$ cells. The relative uniformity of GFP expression, or lack thereof, among the cells within a given sphere indicated that most spheres were clonally derived ($P < 0.005$ by χ^2 analysis). This tested the null hypothesis that the spheres arose from non-clonal aggregation of two or more cells, each of which was equally likely to be GFP$^+$ or GFP$^-$. When the single spheres were plated onto polyornithine and fibronectin and their outgrowth assessed two weeks later, all gave rise to both neurons and glia (Fig. 3*e–g*). Because most secondary spheres were likely to have been clonally derived, and all included neurons as well as glia (38 of 38 spheres; n = 4 samples), single WMPCs must have given rise to neurons and glia together.

We next asked if the neurogenic capacity and multilineage competence of WMPCs were maintained with passage. Primary spheres were raised serially in bFGF/NT3/PDGF-AA for 7 d, DMEM/F12/N1 with 15% serum/PDGF-AA for 4 d, and serum-free DMEM/F12/N1 with bFGF for 10 d. Cells were then dissociated and replated in bFGF at 3,000 cells/well in a 24-well plate. Secondary spheres arose within two weeks from 1.1 ± 0.3% of these cells (n = 8). After more than two weeks of further expansion, the secondary spheres were plated on polyornithine and fibronectin and were fixed and immunostained two weeks later (seven to nine weeks after sorting). Whereas primary spheres consisted of 21.7 ± 4.3% βIII-tubulin$^+$ neurons, 17.7 ± 3.9% glial fibrillary acidic protein (GFAP)$^+$ astrocytes and 46.7 ± 5.9% O4$^+$ oligodendrocytes (n = 3), secondary spheres consisted of 16.0 ± 2.5% neurons, 19.3 ± 3.2% astro-

cytes and 46.4 ± 2.4% oligodendrocytes (n = 3). Most of the neurons were GABAergic, by virtue of their expression of glutamic acid decarboxylase-67 (GAD67) (Fig. 4*a–c*). Because the relative proportions of neurons, oligodendrocytes and astrocytes in secondary spheres were similar to those in primary spheres, we concluded that WMPCs retained multilineage competence with expansion.

WMPC-derived neurons become functionally mature

The calcium responses and membrane currents of WMPC-derived neurons were assessed to establish their ability to respond to depolarizing stimuli. Primary spheres (n = 12 fields, derived from 3 brains) were plated on fibronectin to permit neuronal outgrowth, and assessed 14 d later for their calcium responses to depolarizing stimuli. The cultures were then loaded with the calcium indicator dye Fluo-3 and serially exposed to both 100 μM glutamate and 60 mM potassium during confocal microscopy. Astrocytic responses to depolarization were minimal under these culture conditions, as previously noted. In contrast, neuron-like cells displayed rapid, reversible, >100% elevations in cytosolic calcium in response to potassium, consistent with the activity of neuronal voltage-gated calcium channels (Fig. 4*d–f*). The neuronal phenotype of these cells was then validated by immunostaining for βIII-tubulin.

We then asked whether WMPC-derived neurons would be able to develop the fast sodium currents and action potentials characteristic of electrophysiologically competent neurons. We used whole-cell patch-clamp recording during current stimulation to assess the response of WMPC-derived neurons that arose from plated secondary spheres derived from A2B5-sorted isolates. A total of 58 WMPC-derived fiber-bearing cells were recorded, in 5 cultures derived from 3 patients. Of these, 13 showed voltage-activated sodium ion currents (I_{Na}) of >100 nA, and 7 had I_{Na} > 600, compatible with the fast sodium currents of neuronal depolarization[15,16]. Accordingly, whereas two of five cells with I_{Na} > 800 generated stimulus-evoked action potentials (Fig. 4*g–h*), none did so with I_{Na} < 800. In addition, none of 26 morphologically non-neuronal cells showed substantial (≥100 pA) current-induced sodium currents. Together, these results indicated that neurons arising from adult human WMPCs developed mature electrophysiologic functions, including both fast sodium currents and action potentials.

WMPCs generated neurons without reprogramming

Glial progenitor cells from the postnatal rat optic nerve can generate neurons, under conditions that have been described as 'reprogramming' glial progenitors to multilineage competence[4]. In that study, neurogenesis was achieved by first instructing the cells to an intermediary astrocytic lineage using either serum or bone morphogenetic protein-2, followed by bFGF-stimulated mitogenesis. We asked whether such reprogramming steps are required for the generation of neurons from adult human WMPCs, or whether simple expansion under minimal conditions *in vitro*, with the removal of these cells from their environment, might be sufficient to permit neurogenesis by these cells. Sorted A2B5$^+$ cells were cultured in several permutations of mitogenic and differentiative conditions to identify the minimal conditions permissive for lineage diversification. We compared the phenotypes generated under three conditions: (i) bFGF/NT3/PDGF-AA in SFM (composed of DMEM/F12/N1) for 7 d, followed by 15% FBS/ PDGF-

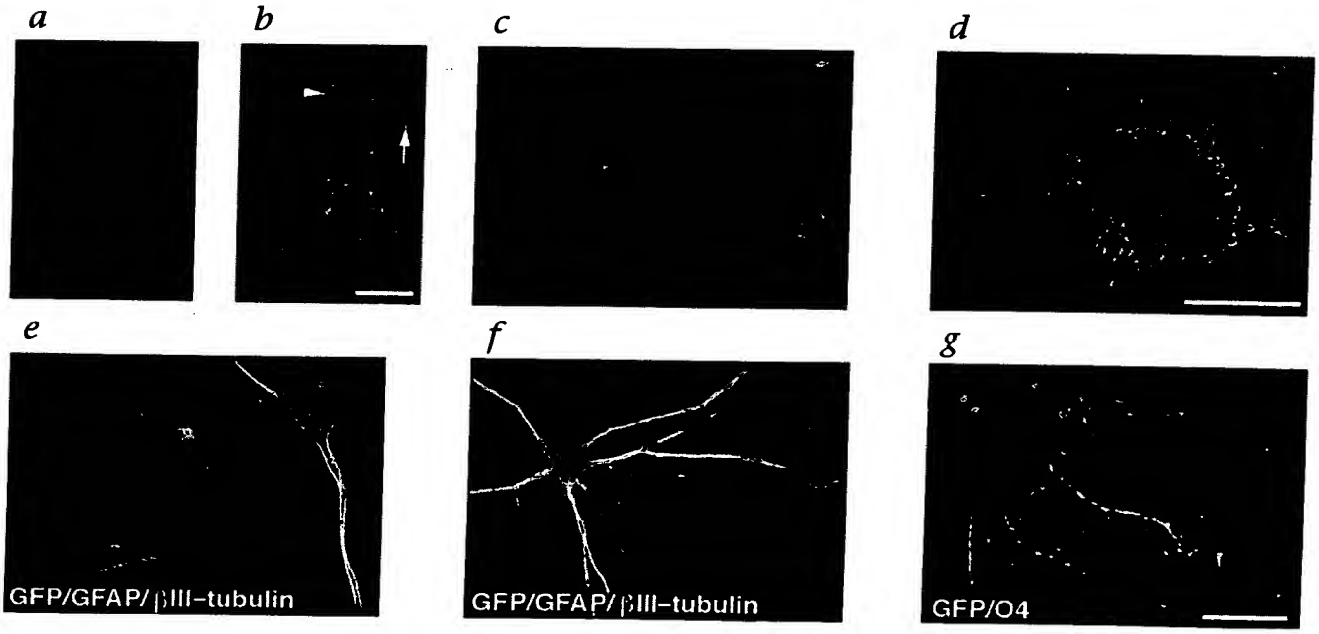

Fig. 3 Single lentiviral GFP-tagged WMPCs generated neurons and glia. A2B5-sorted WMPCs were infected with a lentivirus encoding enhanced GFP[14], 5 d after sorting. ***a*** and ***b***, Secondary spheres subsequently derived from infected cells harbored either GFP-tagged cells (arrowhead), untagged cells (arrow) or, less commonly, both. ***c*** and ***d***, GFP$^+$ secondary sphere 1 week after plating. ***e*** and ***f***, βIII-tubulin$^+$ neurons (red) and GFAP$^+$ astrocytes (blue) arising from a single clonally derived GFP$^+$ secondary sphere. ***g***, GFP$^+$ (green) and O4$^+$ (red) oligodendrocytes arising from a secondary sphere. Scale bars, 100 μm (*a* and *b*), 60 μm (*c* and *d*) or 40 μm (*e–g*).

AA for 4 d and SFM with bFGF for two weeks; (ii) bFGF/NT3/PDGF-AA in SFM continuously for three weeks; and (iii) bFGF alone in SFM for three weeks. The first condition was intended to promote initial differentiation in serum, whereas the latter two groups were designed to skip this glial differentiative step[4].

The A2B5-sorted progenitors yielded spheres under each of these conditions; however, both the number of spheres and the percentage of neurons generated by each differed as a function of treatment. Cultures maintained in base media alone or in bFGF-supplemented media had 5.9 ± 1.7% and 7.2 ± 2.1% βIII-tubulin$^+$ neurons, respectively (n = 3 patients). When matched WMPC-derived spheres were sequentially raised in bFGF/NT3/PDGF-AA with 15% serum and bFGF, 18.2 ± 2.2% of the cells were βIII-tubulin$^+$ (Fig. 5*a*). A similar proportion of neurons (22.5 ± 1.9%; n = 3) was generated by those neurospheres maintained in SFM with bFGF/NT3/PDGF-AA. Serum exposure was therefore not required for A2B5$^+$ cells to generate neurons. Indeed, no specific signals seemed necessary for neuronal instruction, besides those provided by PDGF and NT3. These data indicated that antecedent astrocytic differentiation was not a necessary prerequisite to neurogenesis by adult WMPCs. These cells required neither prolonged mitogenic expansion, nor specific dedifferentiation steps, to generate neurons as well as glia[4].

Although both PDGF and NT3 promote oligodendrocyte production by glial progenitors of the rat optic nerve[17,18], each can induce neuronal differentiation in less-committed hippocampal and ventricular zone neural progenitors[19,20]. As such, their neurogenic effects on adult WMPCs may reflect the relatively undifferentiated state of these cells.

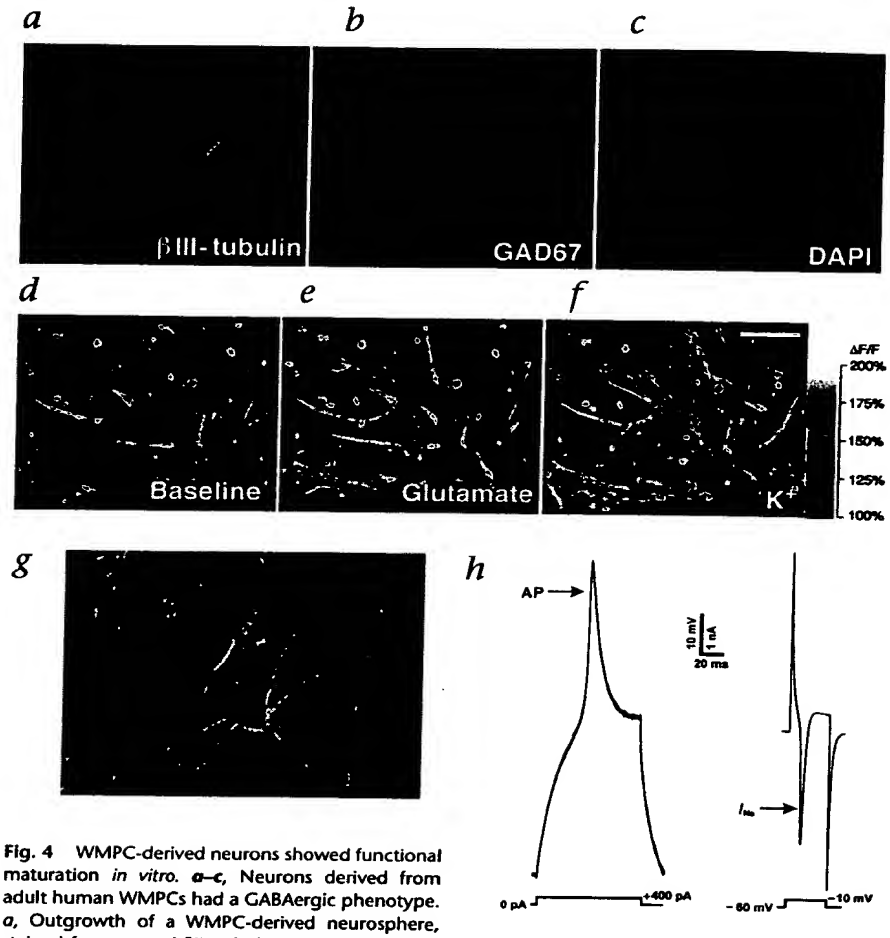

Fig. 4 WMPC-derived neurons showed functional maturation *in vitro*. ***a–c***, Neurons derived from adult human WMPCs had a GABAergic phenotype. *a*, Outgrowth of a WMPC-derived neurosphere, stained for neuronal βIII-tubulin after 35 d *in vitro*. *b*, Immunostaining showed that all 9 neurons in the field were GAD67$^+$ and were thus likely to be GABAergic. *c*, DAPI nuclear counterstaining showed the abundance of cells in the field. ***d–f***, WMPC-derived neurons developed neuronal Ca^{2+} responses to depolarization. *d*, WMPC-derived cells loaded with the calcium indicator dye Fluo-3, 10 d after plating of first-passage spheres derived from A2B5-sorted white matter (35 d *in vitro* total). Many fiber-bearing cells of both neuronal and glial morphologies are apparent. *e*, The same field after exposure to 100 μM glutamate. *f*, The same field after exposure to a depolarizing stimulus of 60 mM KCl. Rapid, reversible, >100% elevations in cytosolic calcium occurred in response to K^+, consistent with the activity of neuronal voltage-gated calcium channels. Scale bar, 80 μm. ***g*** and ***h***, Whole-cell patch-clamp experiments detected voltage-gated sodium currents and action potentials in WMPC-derived neurons. *g*, Representative cell, 14 d after plating of first-passage sphere derived from A2B5-sorted white matter. The cell was patch clamped in a voltage-clamped configuration and its responses to current injection were recorded. *h*, Action potentials (AP) were noted after positive current injection, at I_{Na} >800 pA (left tracing). The fast negative deflections noted after depolarization steps are typical of the voltage-gated sodium currents of mature neurons (right).

Only a fraction of A2B5$^+$ cells were clonogenic

We next assessed the incidence of clonogenic and multipotential progenitor cells within the larger pool of A2B5-sorted white-matter cells. We first assessed whether either the survival or the mitotic competence of adult human WMPCs were dependent on density, by assessing the limiting dilution at which clonogenic progenitors could be obtained from A2B5-sorted white-matter dissociates. A2B5$^+$ cells were plated immediately after sorting, at densities ranging from 100,000 to 1,000 cells/ml (0.5 ml cell suspension per well of a 24-well plate), in basal media supplemented with bFGF/NT-3/PDGF-AA. Under these conditions, the incidence of clonogenic progenitors was a curvilinear function of the sorted cell density (R^2 = 0.978; Fig. 5*b*). Whereas 186 ± 7.6 spheres were generated at a density of 100,000 cells/ml (0.4%; n = 5 patients), only 6.5 ± 2.7 were noted at 10,000 cells/ml (0.1%) and no sphere generation was noted at or below 5,000 cells/ml. Thus, the expansion of purified WMPCs was density dependent and optimal at 50,000–100,000 cells/ml. Densities higher than the optimal range seemed to promote terminal differentiation of the progenitors.

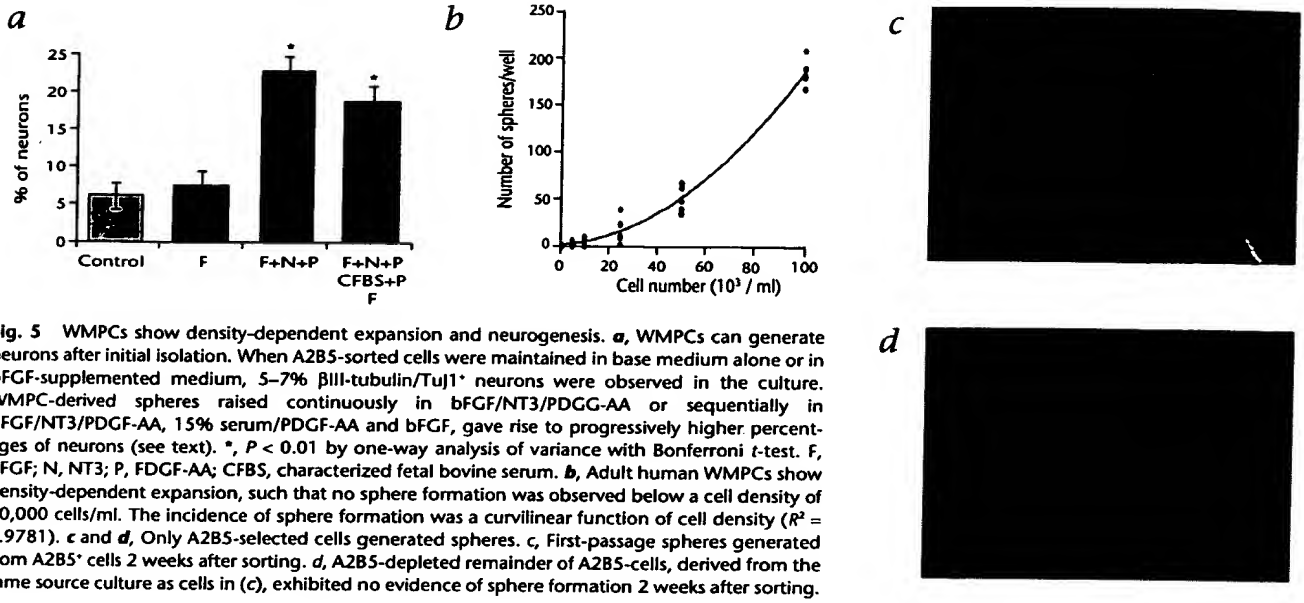

Fig. 5 WMPCs show density-dependent expansion and neurogenesis. ***a***, WMPCs can generate neurons after initial isolation. When A2B5-sorted cells were maintained in base medium alone or in bFGF-supplemented medium, 5–7% βIII-tubulin/TuJ1⁺ neurons were observed in the culture. WMPC-derived spheres raised continuously in bFGF/NT3/PDGG-AA or sequentially in bFGF/NT3/PDGF-AA, 15% serum/PDGF-AA and bFGF, gave rise to progressively higher percentages of neurons (see text). *, $P < 0.01$ by one-way analysis of variance with Bonferroni *t*-test. F, bFGF; N, NT3; P, FDGF-AA; CFBS, characterized fetal bovine serum. ***b***, Adult human WMPCs show density-dependent expansion, such that no sphere formation was observed below a cell density of 10,000 cells/ml. The incidence of sphere formation was a curvilinear function of cell density (R^2 = 0.9781). ***c*** and ***d***, Only A2B5-selected cells generated spheres. *c*, First-passage spheres generated from A2B5⁺ cells 2 weeks after sorting. *d*, A2B5-depleted remainder of A2B5-cells, derived from the same source culture as cells in (*c*), exhibited no evidence of sphere formation 2 weeks after sorting.

To assess whether clonogenic WMPCs were restricted to the A2B5⁺ population, we also cultured the A2B5-depleted pool remaining after each sort. A2B5-depleted cultures did not give rise to any passageable neurospheres at any of the cell densities assessed over the range of 1,000–100,000 cells/ml (Fig. 5*d*). On the basis of these studies, we concluded that only a fraction of white-matter A2B5⁺ cells are actually clonogenic and multipotential progenitors, although all clonogenic WMPCs are A2B5⁺.

Adult WMPCs showed limited self-renewal

We next sought to define the extent to which WMPCs were self-renewing by assessing the extent to which WMPC-derived neurospheres were capable of repetitive passage. Primary spheres were raised from three patients at an optimal initial density of 100,000 cells/ml, under the conditions identified as most supportive of multilineage expansion (bFGF/NT3/PDGF-AA in DMEM/F12/N1). One month later, the spheres were dissociated and replated. Secondary spheres were generated and were replated one month later at 1×10^4–5×10^4 cells/ml. These cultures gave rise to tertiary spheres over the following month, though with less efficiency and a smaller volumetric expansion than secondary spheres. Attempts at propagating these spheres as quaternary spheres, after additional dissociation, were generally unsuccessful. Given an apparent cell doubling time of 3–4 d (data not shown) and monthly passages spanning 8–10 doublings, we estimated that the tertiary spheres assessed one month after the last passage underwent a minimum of 16–24 and no more than 30 doublings. This is well below the number of doublings of which tissue-derived stem cells are typically thought capable.

Our inability to successfully passage these cells beyond 16–24 doublings called into question their ability to self-replicate for extended periods of time *in vitro*. Their limited replicative competence contrasted with that of neural progenitors sorted from the fetal human ventricular zone, which may be readily passaged for >60 doublings under analogous culture conditions[21]. Such self-renewal capacity has been ascribed to sustained telomerase activity in a number of developing systems, including the fetal human forebrain[22,23]. To assess whether the apparently finite proliferative potential of adult human WMPCs reflected a lack of telomerase activity, telomerase levels were assessed using the telomerase reverse transcriptase activity protocol (TRAP) assay[23,24]. We did not detect any telomerase activity in primary or secondary WMPC-derived spheres, despite high-level activity in a variety of positive controls (see Supplementary Fig. 1 online). Their lack of extended replicative potential, coupled with their lack of telomerase activity, suggests that adult WMPCs might constitute a pool of multipotential progenitors with a finite capacity for mitotic expansion, transitional between tissue-restricted stem cells and phenotypically committed progenitors.

WMPCs produced neurons and glia after fetal xenograft

We next assessed whether WMPCs were multipotential *in vivo* as well as *in vitro* by evaluating their fate after engraftment to embryonic stage (E)17 fetal rat brains. Some A2B5-sorted cells were transplanted 24–48 h after sorting to assess their lineage potential upon initial isolation. These cells were maintained only in SFM during the period between isolation and xenograft and were never exposed to any exogenous growth factors. Other cells were transplanted 10 d after sorting, after maintenance in bFGF/NT3/PDGF-AA for 4 d and 15% serum/PDGF-AA followed by bFGF, for 3 days each. All donor cells were administered into E17 rat embryos by intraventricular injection at 10^5 cells/animal. The recipients were killed and fixed four weeks after birth to evaluate the fate of the implanted human cells. Human donor cells were identified by immunolabeling of brain sections for human nuclear antigen (HNA).

In rats implanted with propagated WMPCs (Fig. 6) and their counterparts injected with acutely isolated WMPCs (see Supplementary Fig. 2 online), donor-derived migrants co-expressing HNA with either nestin or doublecortin[25] were found in the host olfactory subpendyma and hippocampus (Fig. 6*a* and

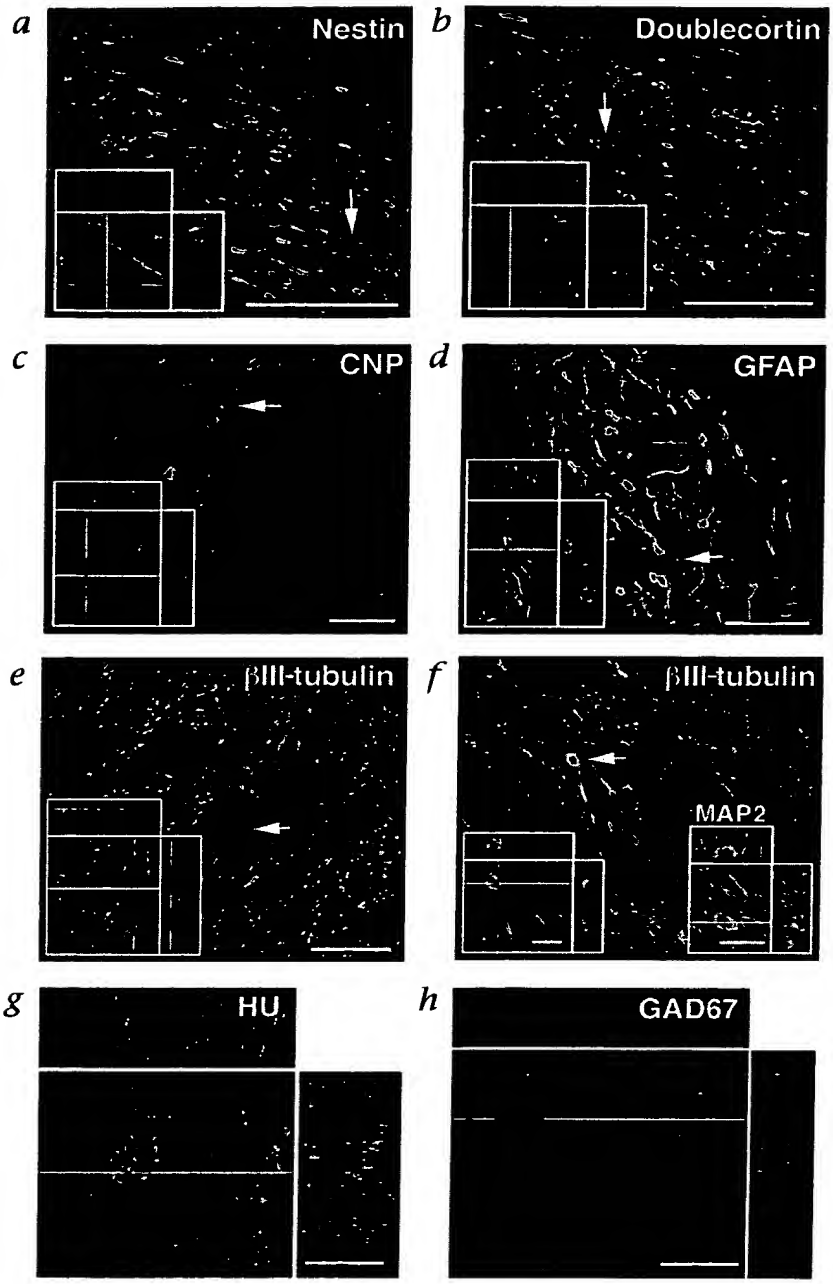

Fig. 6 WMPCs engrafted into fetal rats give rise to neurons and glia in a site-specific manner. Sections from a rat brain implanted at E17 with A2B5-sorted WMPCs and killed 1 month after birth. Cells were maintained in culture for 10 d before implanting. *a* and *b*, Nestin$^+$ (*a*) progenitors and doublecortin$^+$ (*b*) migrants (red) each co-expressing HNA (green) in the hippocampal alvius. *c*, CNP$^+$ (red) HNA$^+$ (green) oligodendrocytes, found exclusively in the corpus callosum. *d*, Low-power image of GFAP$^+$ (green) HNA$^+$ (red) astrocytes (yellow, double-positive) along the ventricular wall. *e*, βIII-tubulin$^+$ (green) and HNA$^+$ (red) neurons migrating in a chain in the hippocampal alvius. *f*, βIII-tubulin$^+$ and MAP2$^+$ (inset) neurons in the striatum, adjacent to the rostral migratory stream (green, bIII-tubulin and MAP2; red, HNA; yellow, double-stained human nuclei). *g*, Hu$^+$ (red) HNA$^+$ (green) neuron in the septum. *h*, GAD67$^+$ (red) HNA$^+$ (green) striatal neuron. Insets (*a–f*) show orthogonal projections of a high-power confocal image of each identified cell (arrow). Scale bars, 40 μm (*a–e*) or 20 μm (*f–h*).

b). In addition, abundant populations of HNA$^+$ βIII-tubulin$^+$ neurons were found in the olfactory subependyma and rostral migratory stream as well as in the hippocampal alvius (Fig. 6*e*). WMPC-derived neurons were also observed in the neostriatum, indicating striatal neuronal differentiation on the part of some xenografted WMPCs (Fig. 6*f*). These data showed that engrafted adult human WMPCs could integrate into the forebrain subventricular zone as neuronal progenitor cells that then gave rise to both granule and striatal neurons. Human WMPC-derived GFAP$^+$ astrocytes and CNP$^+$ oligodendrocytes were also common in recipient brains and were found primarily along the ventricles or in the subcortical white matter (Fig. 6*c* and *d*). Thus, adult human WMPCs showed context-dependent differentiation after xenograft to the developing rat brain and were competent to do so upon acute isolation, without the benefit of humoral instruction *in vitro*.

Discussion

These observations suggest that the WMPCs of the adult human forebrain include multipotential progenitor cells, capable of a finite and limited degree of expansion and self-renewal. These cells remain competent to respond to local instructive cues, with a wide range of lineage choices, upon xenograft as well as *in vitro*. They are readily able to give rise to neurons and glia once they are removed from their native white-matter environment. The freshly isolated adult WMPCs in our study did not require prolonged expansion to undergo neurogenesis *in vitro*, and seemed immediately competent to generate neurons upon xenograft to the developing brain.

Previous studies of the adult rat brain have identified parenchymal progenitor cells that are able to give rise to neurons and glia after a number of cell doublings, in the presence of bFGF[8]. In addition, nominally committed glial progenitor cells derived from the neonatal rat optic nerve have also been reported to give rise to neurons and oligodendrocytes[4]. The lineage diversification of these cells seems to require a humorally directed reprogramming of their phenotype, with the induction of an astrocytic intermediary on the way to neurogenesis. In the present study, adult human WMPCs did not seem to require any such reprogramming or transdifferentiation to achieve multilineage competence. Similarly, they did not seem to pass through an intermediate astrocytic stage before generating neurons, oligodendrocytes and astrocytes. Indeed, after their acute isolation and xenograft, A2B5-defined WMPCs were able to

generate all major neural phenotypes *in vivo* and *in vitro*, without any exogenous growth factor exposure. Nevertheless, because an average of 7% of A2B5-sorted white-matter cells co-expressed GFAP (data not shown), it is possible that some WMPCs exhibit astroglial features at some point during their ontogeny, much like subventricular neural progenitor cells[26,27]. This categorization notwithstanding, our results suggest that the WMPCs of the adult human brain are fundamentally tissue-specific progenitor cells that are tonically restricted to glial lineage by the local parenchymal environment, and do not require specific phenotypic reprogramming for neuronal differentiation.

These data suggest that adult human WMPCs constitute a population of parenchymal glial progenitor cells whose *in situ* fate is restricted by the local white-matter environment. Yet the progenitor cell pool of the adult white matter may be heterogeneous, and it is not clear whether all WMPCs have the same ontogeny or fate potential[28–30]. A minority of multipotential progenitor cells might still persist among a larger pool of more fundamentally lineage-restricted glial progenitors[8]. These parenchymal multipotent progenitors may constitute a relatively rare subpopulation, more akin to persistent stem cells than to any lineage-restricted derivatives[31,32]. In this regard, although we did not detect telomerase activity in sorted WMPCs, if the clonogenic portion of these represents only a small fraction of the total progenitor pool, then their numbers might have been below the detection threshold of our TRAP assay. Further study of the heterogeneity of the white-matter progenitor cell population, and of the lineage competence of its constituent phenotypes, will be needed to define the spectrum of progenitor cell types in the adult brain. These considerations aside, multipotential and neurogenic progenitors are abundant in the adult human white matter and are both extractable and expandable. These cells may prove to be important agents for both induction and implantation strategies of cell-based neurological therapy.

Methods

Tissue dissociation and culture. Adult subcortical white matter was surgically obtained from 21 patients, including 14 undergoing epileptic resections (age 1–50 years; 7 males and 7 females), one undergoing aneurysmal repair (69-year-old male), 2 undergoing resections of a noncontiguous dysplastic focus (20-year-old male and 36-year-old female) and 4 undergoing traumatic temporal lobe decompressions (17–67 years old; all males). Samples were obtained from patients who consented to tissue use under protocols approved by the New York Hospital–Cornell and Columbia Presbyterian Hospital Institutional Review Boards. The samples were dissected and dissociated to single-cell suspensions using papain and DNase as described[2,33,34]. The cells were then suspended in DMEM/F12/N1 with either bFGF (20 ng/ml; Sigma, St. Louis, Missouri) alone or bFGF with NT-3 (2 ng/ml; R&D Minneapolis, Minnesota) and PDGF-AA (20 ng/ml; Sigma), and plated in 100-mm suspension culture dishes (Corning, New York).

Magnetic separation of A2B5$^+$ cells. The number of viable cells was determined using calcein (Molecular Probes, Eugene, Oregon) 24–48 h after dissociation. The cells were then washed and incubated with A2B5 supernatant (clone 105; American Type Culture Collection, Manassas, Virginia) for 30–45 min at 4 °C, washed 3 times with PBS containing 0.5% BSA and 2 mM EDTA, and incubated with microbead-tagged mouse-specific rat IgM (1:4; Miltenyi Biotech, Bergisch Gladbach, Germany) for 30 min at 4 °C. The A2B5$^+$ cells were washed, resuspended and separated using positive selection columns, type MS$^+$ RS$^+$ or LS$^+$ VS$^+$ (magnetic cell sorting (MACS); Miltenyi Biotech). For flow cytometry of matched samples, cells were incubated in FITC-labeled mouse-specific goat IgM at 1:50 before FACS.

Transfection and sorting. Samples were transfected with pCNP2:hGFP after 2–6 d *in vitro*, using 2 μg of plasmid DNA and 10 μl of Lipofectin (Gibco, Carlsbad, California) as described[2,33,35]. Sorting for pCNP2:hGFP and A2B5 immunofluorescence was performed on a Becton-Dickinson FACS Vantage (San Diego, California), also as described[2,33,35]. Untransfected and IgM-exposed control cells were used to calibrate background; a false-positive rate of 1% was accepted as cutoff.

Generation of primary and secondary spheres. A2B5$^+$ and A2B5-depleted white-matter cells were distributed to a 24-well plate directly after sorting, at 100,000, 50,000, 25,000, 10,000, 5,000 and 1,000 cells/ml with 0.5 ml/well of DMEM/F12/N1 with bFGF/NT3/PDGF-AA. The resulting WMPC-derived neurospheres were passaged at the 50- to 100-cell stage, by dissociation to single cells with trypsin and EDTA. The cells were plated at 3,000 cells/well. Three weeks later, the resultant secondary spheres were either dissociated and passaged again as tertiary spheres, or plated into 2% FBS with 20 ng/ml brain-derived neurotrophic factor on a polyornithine and fibronectin substrate and fixed 2 weeks later.

Lentiviral tagging and lineage analysis. A2B5-sorted cells were infected 2–5 d after separation with lentivirus (10^8 PFU/ml) expressing GFP under CMV promoter control and a WPRES post-transcriptional regulatory element[12,13]. The lentivirus was generated by co-transfecting plasmids pCMV/DR8.91, pMD.G, and pHRCMVGFPwsin into 293T cells as described[14]. A2B5-sorted cells were exposed to lentivirus for 24 h in polybrene-supplemented medium (8 μg/ml), then passaged into fresh medium in 24-well plates. GFP expression by tagged cells was observed within 2 d. The primary spheres that arose in these cultures were dissociated 3 weeks later and replated at 3,000 cells/well; secondary spheres arose from these within 2 weeks.

TRAP assay. Telomerase activity was determined using the TRAP assay[23,24], described in detail in the material accompanying Supplementary Figure 1 online.

***In utero* transplantation.** Transuterine xenograft into E17 rat fetuses was performed as described[21,36]. Some cells were injected within 24–48 h after sorting and others after 10 d *in vitro* in FGF2, PDGF-AA and NT3. One month after implantation, the animals were perfusion-fixed by 4% paraformaldehyde. Experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University.

Immunocytochemistry. Xenografted rat brains were cryosectioned at 15 μm, permeabilized with PBS, 0.1% saponin and 1% NGS, and blocked with PBS, 0.05% saponin and 5% NGS, each for 30 min. Sections were labeled with HNA-specific mouse antibody (1:50; Chemicon, Temecula, California), then immunostained with βIII-tubulin–specific antibody TuJ1 (1:600; Covance, Princeton, New Jersey), MAP2-specific antibody AP-20 (1:50; Sigma), HuC/HuD-specific mouse monoclonal antibody 16A11 (25 μg/ml; H. Furneaux, Memorial Sloan-Kettering Cancer Center, New York), GAD67-specific rabbit antibody (1:100; Chemicon), GFAP-specific mouse antibody SMI 21 (1:1,000; Sternberger, Lutherville, Maryland), GFAP-specific rabbit antibody (1:400; Sigma), CNP-specific mouse antibody SMI 91 (1:1000 Sternberger), human nestin–specific rabbit antibody (1:200; Chemicon), or doublecortin-specific rabbit antisera (1:100; C. Walsh, Harvard Medical School, Boston, Massachusetts). The sections were incubated with antibody overnight at 4 °C. Species- and isotype-specific fluorescent secondary antibodies were applied at 1:100 for 1.5 h at room temperature.

O4 and A2B5 were immunolabeled *in vitro* as described[2]. For multiple-antigen labeling, O4 was localized on live cells that were then fixed and stained for βIII-tubulin, MAP2, GFAP, Hu, GAD67 or BrdU. O4 supernatant (R. Bansal and S. Pfeiffer, University of Connecticut Health Center, Farmington, Connecticut) was used at 1:100 for 40 min at 4 °C. Antibodies against βIII-tubulin, MAP-2, GFAP and BrdU (BrdU-specific rat antibody; 1:200; Harlan, Indianapolis, Indiana) were incubated overnight at 4 °C. Fixed cultures were counterstained with DAPI (10 μg/ml; Molecular Probes).

Confocal imaging. In the xenografted brains, single cells that appeared co-labeled for both human- and cell-specific markers were evaluated by confocal imaging as described[21,37]. To be deemed double labeled, cells were

required to have HNA-specific signal surrounded by neuronal or glial immunoreactivity in every serially acquired 0.4-μm *z*-dimension optical section, as well as in each orthogonal side view thereof.

Calcium imaging. Outgrowths from both first- and second-passage WMPC-derived neurospheres were assessed 2–3 weeks after plating into BDNF-supplemented DMEM/F12/N1 with 2% FBS. These mixed neuronal and glial outgrowths were challenged with 100 μM glutamate or 60 mM potassium. Cytosolic calcium imaging was conducted using confocal microscopy of cultures loaded with Fluo-3 acetoxymethylester (Molecular Probes)[33,38,39]. We previously reported that adult progenitor–derived human neurons showed a mean calcium rise of >400% in response to 60 mM potassium *in vitro*, compared with glial responses of <20%[38]. In this study, we assigned neuronal identity to cells with ≥2-fold calcium elevations to depolarization.

Electrophysiology. Sister cultures to those subjected to calcium imaging were assessed by whole-cell patch-clamp analysis. Whole-cell voltage-clamped recordings of fiber-bearing cells were conducted and analyzed as described[15,33]. A holding potential of –60 mV and voltage steps of 10 mV with 100-ms durations were applied to the recorded cells through the patch electrodes. Signals were sampled every 50 μs.

Note: Supplementary information is available on the Nature Medicine *website.*

Acknowledgments

This study was supported by US National Institute of Neurological Disorders and Stroke grants R01NS39559, R01NS33106 and R37/R01NS29813, and by the National Multiple Sclerosis Society, Christopher Reeve Paralysis Foundation, and Project ALS. N.S.R. and M.N. were supported in part by the American Heart Association. M.N. was supported by the Fundação para a Ciencia e Tecnologia and the Gulbenkian Foundation of Portugal. We thank D. Trono for the lentiviral plasmids; C. Walsh for anti-doublecortin antisera; H. Furneaux for Hu-specific monoclonal antibody 16A11; R. Bansal and S. Pfeiffer for the monoclonal O4 line; and A. Benraiss, M. Windrem and T. Takano for advice and assistance.

Competing interests statement

The authors declare that they have no competing financial interests.

RECEIVED 4 DECEMBER 2002; ACCEPTED 19 FEBRUARY 2003

1. Scolding, N. *et al.* Oligodendrocyte progenitors are present in the normal adult human CNS and in the lesions of multiple sclerosis [see comments]. *Brain* **121**, 2221–2228 (1998).
2. Roy, N.S. *et al.* Identification, isolation, and promoter-defined separation of mitotic oligodendrocyte progenitor cells from the adult human subcortical white matter. *J. Neurosci.* **19**, 9986–9995 (1999).
3. Gravel, M., DiPolo, A., Valera, P. & Braun, P. Four-kilobase sequence of the mouse CNP gene directs spatial and temporal expression of lacZ in transgenic mice. *J. Neurosci. Res.* **53**, 393–404 (1998).
4. Kondo, T. & Raff, M. Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. *Science* **289**, 1754–1757 (2000).
5. Palmer, T.D., Markakis, E.A., Willhoite, A.R., Safar, F. & Gage, F.H. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J. Neurosci.* **19**, 8487–8497 (1999).
6. Reynolds, B.A. & Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707–1710 (1992).
7. Vescovi, A.L., Reynolds, B.A., Fraser, D.D. & Weiss, S. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron* **11**, 951–966 (1993).
8. Morshead, C.M. *et al.* Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron* **13**, 1071–1082 (1994).
9. Vescovi, A. *et al.* Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human stem cells lines by epigenetic stimulation. *Exp. Neurol.* **156**, 71–83 (1999).
10. Svendsen, C., Caldwell, M. & Ostenfeld, T. Human neural stem cells: isolation, expansion and transplantation. *Brain Pathol.* **9**, 499–513 (1999).
11. Carpenter, M. *et al. In vitro* expansion of a multipotent population of human neural progenitor cells. *Exp. Neurol.* **158**, 265–278 (1999).
12. Han, J. *et al.* Transgene expression in the guinea pig cochlea mediated by a lentivirus-derived gene transfer vector. *Hum. Gene Ther.* **10**, 1867–1873 (1999).
13. Zufferey, R., Donello, J., Trono, D. & Hope, H. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73**, 2886–2892 (1999).
14. Windrem, M. *et al.* Progenitor cells derived from the adult human subcortical white matter disperse and differentiate as oligodendrocytes within demyelinated regions of the rat brain. *J. Neurosci. Res.* **69**, 966–975 (2002).
15. Kang, J., Jiang, L., Goldman, S.A. & Nedergaard, M. Astrocyte-mediated potentiation of inhibitory synaptic transmission. *Nat. Neurosci.* **1**, 683–692 (1998).
16. Tse, F.W., Fraser, D.D., Duffy, S. & MacVicar, B.A. Voltage-activated K^+ currents in acutely isolated hippocampal astrocytes. *J. Neurosci.* **12**, 1781–1788 (1992).
17. Barres, B.A. *et al.* A crucial role for neurotrophin-3 in oligodendrocyte development. *Nature* **367**, 371–375 (1994).
18. Raff, M.C., Lillien, L.E., Richardson, W.D., Burne, J.F. & Noble, M.D. Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature* **333**, 562–565 (1988).
19. Vicario-Abejon, C., Johe, K.K., Hazel, T.G., Collazo, D. & McKay, R.D. Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. *Neuron* **15**, 105–114 (1995).
20. Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M. & McKay, R.D. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev.* **10**, 3129–3140 (1996).
21. Keyoung, H.M. *et al.* Specific identification, selection and extraction of neural stem cells from the fetal human brain. *Nat. Biotechnol.* **19**, 843–850 (2001).
22. Yashima, K. *et al.* Expression of the RNA component of telomerase during human development and differentiation. *Cell Growth Differ.* **9**, 805–813 (1998).
23. Ostenfeld, T. *et al.* Human neural precursor cells express low levels of telomerase *in vitro* and show diminishing cell proliferation with extensive axonal outgrowth following transplantation. *Exp. Neurol.* **164**, 215–226 (2000).
24. Kim, N. *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015 (1994).
25. Gleeson, J., Lin, P., Flanagan, L. & Walsh, C. Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron* **23**, 257–271 (1999).
26. Doetsch, F., Caille, I., Lim, D., Garcia-Verdugo, J. & Alvarez-Buylla, A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**, 703–716 (1999).
27. Alvarez-Buylla, A. & Garcia-Verdugo, J.M. Neurogenesis in adult subventricular zone. *J. Neurosci.* **22**, 629–634 (2002).
28. Kukekov, V., Laywell, E., Thomas, L. & Steindler, D. A nestin-negative precursor cell from the adult mouse brain gives rise to neurons and glia. *Glia* **21**, 399–407 (1997).
29. Levine, J.M., Reynolds, R. & Fawcett, J.W. The oligodendrocyte precursor cell in health and disease. *Trends Neurosci.* **24**, 39–47 (2001).
30. Mason, J. & Goldman, J. A2B5$^+$ and O4$^+$ cycling progenitors in the adult forebrain white matter respond differentially to PDGF-AA, FGF-2, and IGF-1. *Molec. Cell. Neurosci.* **20**, 30–42 (2002).
31. Jiang, Y. *et al.* Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41–49 (2002).
32. Capela, A. & Temple, S. LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. *Neuron* **35**, 865–875 (2002).
33. Roy, N.S. *et al. In vitro* neurogenesis by progenitor cells isolated from the adult human hippocampus. *Nat. Med.* **6**, 271–277 (2000).
34. Roy, N.S. *et al.* Promoter-targeted selection and isolation of neural progenitor cells from the adult human ventricular zone. *J. Neurosci. Res.* **59**, 321–331 (2000).
35. Wang, S. *et al.* Isolation of neuronal precursors by sorting embryonic forebrain transfected with GFP regulated by the T α 1 tubulin promoter. *Nat. Biotechnol.* **16**, 196–201 (1998).
36. Brustle, O. *et al.* Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats. *Nat. Biotechnol.* **16**, 1040–1044 (1998).
37. Benraiss, A., Chmielnicki, E., Roh, D. & Goldman, S.A. Adenoviral BDNF induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. *J. Neurosci.* **21**, 6718–6731 (2001).
38. Kirschenbaum, B. *et al. In vitro* neuronal production and differentiation by precursor cells derived from the adult human forebrain. *Cereb. Cortex* **4**, 576–589 (1994).
39. Pincus, D.W. *et al.* FGF2/BDNF-associated maturation of new neurons generated from adult human subependymal cells. *Ann. Neurol.* **43**, 576–585 (1998).
40. Marusich, M., Furneaux, H., Henion, P. & Weston, J. Hu neuronal proteins are expressed in proliferating neurogenic cells. *J. Neurobiol.* **25**, 143–155 (1994).
41. Barami, K., Iversen, K., Furneaux, H. & Goldman, S.A. Hu protein as an early marker of neuronal phenotypic differentiation by subependymal zone cells of the adult songbird forebrain. *J. Neurobiol.* **28**, 82–101 (1995).

nature medicine

Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain

Martha S Windrem[1], Marta C Nunes[1], William K Rashbaum[2], Theodore H Schwartz[3], Robert A Goodman[4], Guy McKhann II[4], Neeta S Roy[1] & Steven A Goldman[1,5]

Both late-gestation and adult human forebrain both contain large numbers of oligodendrocyte progenitor cells (OPCs). These cells may be identified by their A2B5$^+$PSA-NCAM$^-$ phenotype (positive for the early oligodendrocyte marker A2B5 and negative for the polysialylated neural cell adhesion molecule). We used dual-color fluorescence-activated cell sorting (FACS) to extract OPCs from 21- to 23-week-old fetal human forebrain, and A2B5 selection to extract these cells from adult white matter. When xenografted to the forebrains of newborn shiverer mice, fetal OPCs dispersed throughout the white matter and developed into oligodendrocytes and astrocytes. By 12 weeks, the host brains showed extensive myelin production, compaction and axonal myelination. Isolates of OPCs derived from adult human white matter also myelinated shiverer mouse brain, but much more rapidly than their fetal counterparts, achieving widespread and dense myelin basic protein (MBP) expression by 4 weeks after grafting. Adult OPCs generated oligodendrocytes more efficiently than fetal OPCs, and ensheathed more host axons per donor cell than fetal cells. Both fetal and adult OPCS phenotypes mediated the extensive and robust myelination of congenitally dysmyelinated host brain, although their differences suggested their use for different disease targets.

A broad range of pediatric leukodystrophies and storage diseases manifest with myelin failure or loss. Recent studies have focused on the use of transplanted oligodendrocytes or their progenitors to treat congenital myelin diseases. The myelinogenic potential of implanted brain cells was first noted in the shiverer mouse[1,2]. Shiverer is an autosomal recessive mutation; *shi/shi* homozygotes fail to develop MBP or compact myelin and die by 20–22 weeks. Transplanted fetal brain cells[3–6], primary[7] and immortalized[8] neural progenitors, and enriched glial progenitor cells[9] can all myelinate shiverer axons, albeit typically with low efficiency. Similarly, rodent subventricular zone progenitors can engraft another dysmyelinated mutant, the myelin-deficient rat, after perinatal administration[10,11]. Indeed, all of these studies suggest the feasibility of myelinating congenitally dysmyelinated brain, even though none of the cell sources used did so efficiently.

On this basis, we asked whether highly enriched populations of OPCs directly isolated from the human brain might be used as more effective substrates for cell-based therapy of congenital dysmyelination. Specifically, we postulated that human OPCs, whether derived from the fetal brain during its period of maximum oligoneogenesis, or from the adult subcortical white matter[12,13], could mediate large-scale myelination of a congenitally dysmyelinated host. We report here that both fetal and adult human OPCs, highly enriched by surface antigen–based FACS, were capable of widespread and high-efficiency myelination of the shiverer mouse brain after perinatal xenograft. We also report significant differences in the behavior of fetal and adult-derived OPCs, which suggests that they may be useful in treating different specific disease targets.

Cells dissociated from the late second-trimester human ventricular zone (21–23 weeks gestation) were first magnetically sorted to isolate A2B5$^+$ cells[13–16], including oligodendrocytic and neuronal progenitor cells. Because PSA-NCAM is expressed by immature neurons at this stage of development[17], we then used FACS to deplete PSA-NCAM$^+$ neurons from the larger A2B5$^+$ cell population. This yielded a subpopulation of A2B5$^+$PSA-NCAM$^-$ cells, which defined our oligodendrocyte progenitor pool. Two-color FACS showed that the A2B5$^+$PSA-NCAM$^-$ fraction constituted 15.4 ± 4.8% of the cells in samples from the 21- to 23-week ventricular zone (n = 5; Supplementary Figure 1 online). Of these A2B5$^+$PSA-NCAM$^-$ cells, 76.1 ± 0.5% expressed oligodendrocytic O4 by 1 week after FACS, whereas only 7.5 ± 0.3% expressed astrocytic glial fibrillary acidic protein (GFAP) and only 2.0 ± 1.3% expressed neuronal β-III tubulin. These data support the glial restriction and oligodendrocytic bias of sorted A2B5$^+$PSA-NCAM$^-$ cells. Because we achieved higher net yields with immunomagnetic separation of A2B5$^+$ cells followed by FACS depletion of NCAM$^+$ cells, compared with two-color FACS, we used this technique for progenitor isolation.

Homozygous *shi/shi* mice were injected intracallosally with fetal progenitor cell isolates on either their day of birth (P0) or on postnatal day 1 (P1), and later killed at 4, 8, 12 or 16 weeks of age. None of the animals were immunosuppressed; we relied on perinatal tolerization to ensure graft acceptance[18,19]. The injections resulted in substantial engraftment, defined as ≥100 cells per coronal section at three rostrocaudal levels sampled >100 mm apart, in 34 of the 44 neonatal mice injected for this study (25 of 33 injected with fetal human OPC, and 9 of the 11 injected with adult-derived OPCs). By 12 weeks of age, the recipients showed donor engraftment throughout the callosum and capsular and commissural

[1]Departments of Neurology and Neuroscience, [2]Obstetrics and Gynecology and [3]Neurosurgery, Cornell University Medical College, 1300 York Ave., New York, New York 10021, USA. [4]Department of Neurosurgery, Columbia University Medical School, 710 W. 160th St., New York, New York 10032, USA. [5]Department of Neurology, University of Rochester Medical Center, 601 Elmwood Ave., Box 645, Rochester, New York 14642, USA. Correspondence should be addressed to S.A.G. (steven_goldman@urmc.rochester.edu).

Published online 21 December 2003; doi:10.1038/nm974

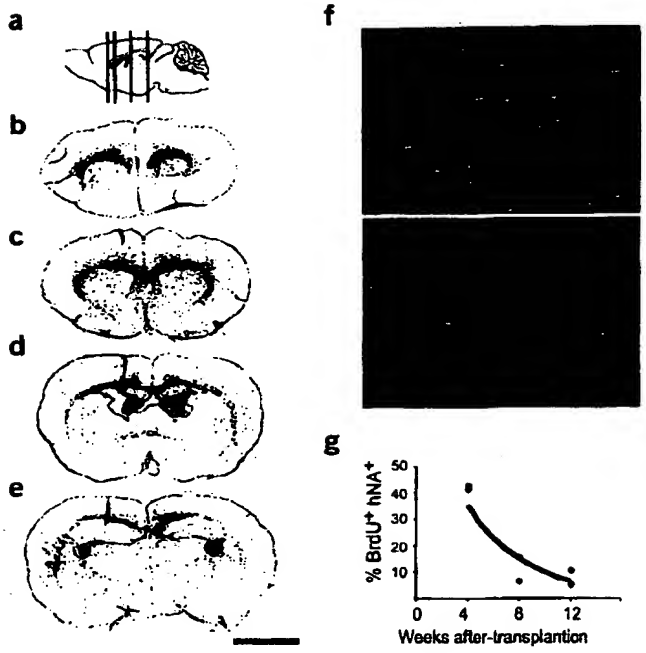

Figure 1 Fetal human OPCs disperse rapidly to infiltrate the forebrain. (**a–e**) Human cells were localized by immunostaining for hNA. Low-power fluorescence images of coronal section of forebrain(**b–e**) were collected at representative anteroposterior levels as indicated in schematic (**a**; ref.25) Engrafted cells are shown in red (**b–e**). (**f**) Immunofluorescent detection of BrdU (green) and hNA (red) 4 (top) and 12 (bottom) weeks after xenograft of human OPCs into shiverer mice. Arrows indicate mitotic human OPCs ($BrdU^+hNA^+$). (**g**) Regression plot of mitotically active donor cells as a function of time after perinatal implant. Rate of BrdU incorporation declined according to the exponential regression $y = 83.4e^{-0.22x}$, with correlation coefficient $r = -0.87$ ($P = 0.012$). Scale bar, 3 mm (**b–e**) or 50 μm (**f**).

white matter, extending caudally to the basis pontis (Fig. 1a–e). During this time, cell division among the engrafted progenitors, though initially high at 4 weeks, fell to relatively low and stable levels by 8 and 12 weeks (Fig. 1f,g). The fraction of human donor cells that incorporated BrdU during the 48 h before mice were killed dropped from 42 ± 6.1% at 4 weeks to 8.2 ± 2.4% at 12 weeks.

During this same period, many of the fetal progenitors matured into myelinogenic oligodendrocytes, as indicated by their expression of MBP. At 4 weeks, no MBP was detectable in 10 of 11 animals, despite widespread cell dispersion; scattered MBP^+ cells were noted in one mouse. At 8 weeks, patchy foci of MBP expression were noted in four of seven mice, and by 12 weeks, widespread MBP expression was noted throughout the forebrain white matter tracts in five of seven mice. By this time, the engrafted mice typically expressed MBP throughout the entire corpus callosum, as well as throughout the fimbria and internal capsules (Fig. 2a–d). Because shiverer mice express only the first exon of the *Mbp* gene[2], and hence have no immunodetectable MBP, any MBP detected in these recipients was necessarily donor-derived[8]. In addition, optical sectioning confirmed that the MBP^+ cells were of human origin, in that each MBP^+ profile was associated with a human nuclear antigen $(hNA)^+$ soma (Fig. 2c,e–h).

We next asked whether donor-derived myelin effectively wrapped host axons. We used confocal imaging and electron microscopy to assess axonal ensheathment and myelin compaction, respectively. Confocal analysis was first done on the brains of three shiverer mice that were implanted on P1 with 100,000 fetal human OPCs each, and sacrificed at 12 weeks. Foci of dense MBP expression were assessed by confocal imaging, after immunolabeling for hNA and neurofilament (NF) protein to detect donor-derived cells and host shiverer axons, respectively. We found

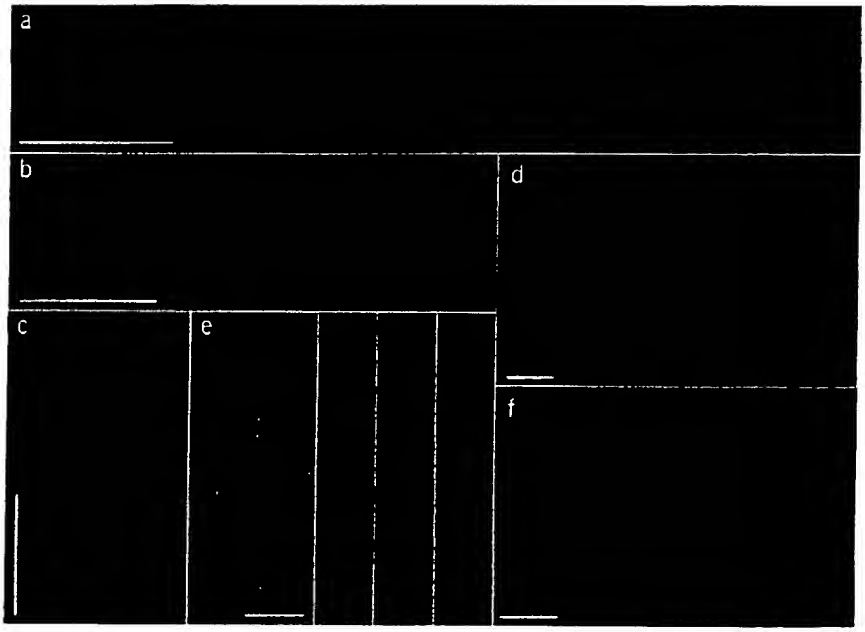

Figure 2 Engrafted human OPCs myelinate an extensive region of the forebrain. (**a,b**) MBP expression (green) by sorted human fetal OPCs implanted into homozygous shiverer mice. Large regions of the corpus callosum were myelinated by 12 weeks. **a** and **b** are two different mice. (**c**) Human OPCs migrated to and myelinated fibers throughout the dorsoventral extents of the internal capsules, resulting in widespread forebrain remyelination after a single perinatal injection. (**d**) MBP expression (green) in engrafted shiverer mouse callosum 3 months after perinatal xenograft was associated with human hNA^+ donor cells (red). (**e**) Confocal optical sections of implanted shiverer mouse callosum, with hNA^+ donor cells (red) surrounded by MBP (green). Human cells (arrows) were found within meshwork of MBP^+ fibers. Right three images, taken 1 μm apart, were merged to form left image. (**f**) Striatocallosal border of shiverer mouse brain, 3 months after perinatal engraftment with human fetal OPCs (blue). Donor-derived MBP^+ oligodendrocytes and myelin (red) are evident in the corpus callosum, while donor-derived $GFAP^+$ astrocytes (green) predominate on the striatal side. Scale bar, 1 mm (**a–c**), 100 μm (**d**), 20 μm (**e**) or 200 μm (**f**).

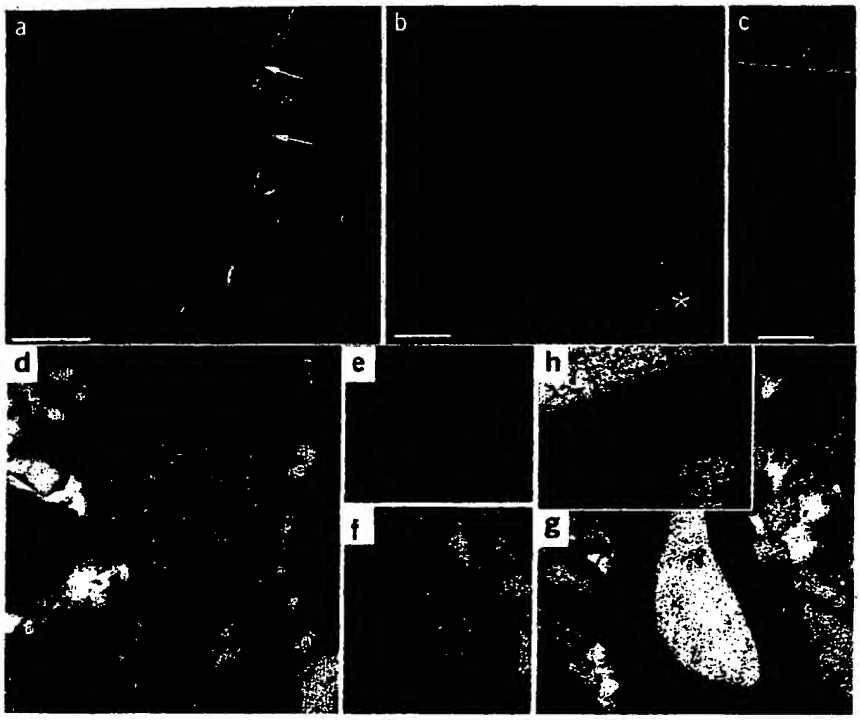

Figure 3 Axonal ensheathment and myelin compaction by engrafted human OPCs. (**a**) Confocal micrograph showing triple immunostain for MBP (red), human nuclear antigen (HNA; blue) and NF (green). All MBP immunostaining is derived from sorted human OPCs, whereas NF$^+$ axons are those of mouse host. Arrows indicate segments of mouse axons ensheathed by human oligodendrocytic MBP. (**b**) Composite of optical sections through corpus callosum of shiverer recipient killed 12 weeks after fetal OPC implantation. (**c**) Higher magnification of area indicated by * in **b**. MBP immunoreactivity (red) surrounds ensheathed axons (green) on both sides. (**d**) Electron micrographs of sagittal section through corpus callosum of adult *shi/shi* homozygote. Shiverer axons typically have single loose wrapping of uncompacted myelin, such that major dense lines do not form. (**e–h**) Representative electron micrographs of 16-week-old homozygous shiverer mice implanted with human OPCs shortly after birth. These images show resident shiverer axons with densely compacted myelin sheaths. **h**, enlargement of area indicated by * in **g**. Major dense lines are visible between myelin lamellae, providing electron microscopic confirmation of myelination by engrafted human OPCs. Scale bar, 20 μm (**a**,**b**), 5 μm (**c**) or 1 μm (**d–h**); **d**, **f**, **g** use bar in **e**.

that the human progenitors generated myelinating oligodendrocytes in great numbers. Of the recipients scored, 11.9 ± 1.6% (mean ± s.e.m.) of NF$^+$ host callosal axons were surrounded by MBP immunoreactivity (n = 3 mice; three fields scored per animal; Fig. 3a–c). We next used electron microscopy to verify that host axons were fully ensheathed by donor-derived oligodendrocytes, and that the latter generated compact myelin. Because MBP is required to compact consecutive layers of myelin together, its expression is required for the major dense line of mature myelin. Myelin in MBP-deficient shiverer mice did not show more than a few loose wrappings and lacked major dense lines (Fig. 3d), whereas *shi/shi* graft recipients showed compact myelin with major dense lines (Fig. 3e–h). In a sample of MBP$^+$ fields (n = 50) derived from two mice killed 16 weeks after perinatal implant, 7.4% of callosal axons (136 of 1,832 sampled) had donor-derived myelin sheaths, as defined ultrastructurally by their major dense lines. Thus, engrafted fetal human OPCs efficiently differentiated into myelinogenic oligodendrocytes.

Some transplanted fetal OPCs differentiated into GFAP$^+$ astrocytes as early as 4 weeks after implantation. In white-matter regions sampled on the basis of high donor-cell engraftment, 12.7 ± 4.3% of fetal donor-derived cells expressed astrocytic GFAP at 12 weeks, and 10.2 ± 4.4% of donor cells expressed MBP. No heterotopic β-III tubulin- or MAP-2-defined neurons of donor derivation were noted at 4, 8 or 12 weeks after implant (n = 33 total). Nevertheless, 40.3 ± 4.2% of donor cells expressed S100-β, which is expressed by astrocytes and young oligodendrocytes, and nestin was expressed by 47.3 ± 4.2%, suggesting that a large proportion of donor cells persisted as glial progenitors after engraftment. Fetal OPCs were recruited as oligodendrocytes or astrocytes in a context-dependent manner, giving rise to both oligodendrocytes and fibrous astrocytes in the presumptive white matter, but only to GFAP$^+$ astrocytes in the gray matter (Fig. 2f and Supplementary Fig. 2 online).

We next asked whether adult-derived OPCs differed from their fetal counterparts with respect to their dispersal, myelinogenic capacity, or time courses thereof. We implanted two litters of P0 shiverer mice with A2B5-sorted OPCs extracted from adult human subcortical white matter. The mice were killed after 4, 8 or 12 weeks, and their brains were stained for hNA and either MBP or GFAP. Nine of 11 mice were successfully engrafted. The adult OPCs achieved widespread and dense MBP expression by 4 weeks (Fig. 4a–d); at 12 weeks, 39.5 ± 16.3% of adult OPCs expressed MBP. In contrast, none of the hNA$^+$ fetal donor OPCs expressed MBP 4 weeks after engraftment, and only 10.2 ± 4.4% did so by 12 weeks ($P < 0.001$ by two-tailed t-test comparing the proportion of MBP$^+$ cells in fetal and adult-derived grafts; Fig. 4a–c). These results indicate that engrafted adult OPCs were at least four times more likely to become oligodendrocytes and develop myelin than their fetal counterparts. Essentially no adult OPCs became astrocytes in the recipient white matter (none developed GFAP expression), whereas 12.7 ± 4.3% of fetal OPCs did so by 12 weeks. Thus, whereas nominally oligodendrocytic progenitors derived from the fetal brain acted as glial progenitors, adult OPCs behaved in a more restricted manner, largely generating either myelinogenic oligodendrocytes or persistent progenitors in recipient white matter. The more rapid myelination by adult OPCs was reflected ultrastructurally, as the major dense lines of compact myelin were readily evident in mice 6 weeks after implantation with adult OPCs at birth (Fig. 4e). No such evidence of myelin compaction was noted in mice implanted with fetal OPCs until 12–16 weeks postnatally.

Despite the apparent competitive advantage of adult OPCs, substantially more fetal than adult donor cells became engrafted in the recipient brains (Fig. 4f). At the midline of the corpus callosum, the region of maximal engraftment, we scored 1,123 ± 205.6 hNA$^+$ fetal donor cells/mm^2. Of these, 117 ± 43.7 were MBP$^+$, and 9.8 ± 3.1% of fetal donor cells dif-

ferentiated into myelinating oligodendrocytes by 12 weeks. In contrast, only 244 ± 182.1 donor cells/mm^2 were noted in the callosal midline of shiverer mice implanted with adult OPCs. Yet 81 ± 59.7, or 38.9 ± 12.9%, of these cells had developed into MBP$^+$ oligodendrocytes by 12 weeks ($P < 0.001$ by two-tailed t-test comparing the proportion of MBP$^+$ cells in fetal and adult grafts; Fig. 4g). In addition, whereas 12.7 ± 4.3% of fetal donor cells matured to express GFAP, no adult donor cells gave rise to GFAP$^+$ astrocytes, again suggesting a stronger bias toward the oligodendrocytic phenotype by the adult progenitors. Thus, besides maturing more quickly than fetal OPCs, adult OPCs gave rise to oligodendrocytes in much higher proportions than their fetal counterparts.

To assess whether adult and fetal OPCs differ in the extent to which they ensheath axons, we scored the numbers of axons myelinated by each donor OPC, as defined by confocal-verified MBP$^+$ wrapping of NF$^+$ axons. These absolute values were then expressed as ratios to total number of donor cells and to donor-derived MBP$^+$ oligodendrocytes per field. When assessed 12 weeks after perinatal graft, adult-derived OPCs ensheathed many more host axons per donor cell than their fetal counterparts, an effect that persisted even after we limited our analysis to the number of ensheathed axons per MBP$^+$ donor cell (Fig. 4h). In each case, the difference between fetal and adult donor ensheathment efficiency was significant by Mann-Whitney analysis ($P < 0.02$). Thus, adult-derived OPCs matured to ensheath more axons per donor cell than their fetal counterparts.

These results indicate that isolates of human OPCs sorted from the highly oligoneogenic, late second-trimester forebrain, as well as from adult subcortical white matter, can broadly myelinate the shiverer mouse brain, a genetic model of perinatal leukodystrophy. When introduced as highly enriched isolates, both fetal and adult-derived OPCs spread widely throughout the presumptive white matter, ensheathed resident mouse axons and formed antigenically and ultrastructurally compact myelin. Donor-derived myelinogenesis was geographically extensive and was observed throughout all white matter regions of the telencephalon. After implantation, the mitotic expansion of the cells slowed over time (Fig. 1g), and neither undesired phenotypes nor parenchymal aggregates were generated. Both fetal and adult-derived OPCs were capable of remyelinating mouse axons, and neither generated heterotopic neurons. We also noted some marked differences between fetal and adult-derived OPCs. Whereas fetal OPCs were highly migratory, they myelinated slowly and inefficiently, and cogenerated astrocytes in recipient white matter as readily as they did myelinogenic oligodendrocytes. In contrast, adult OPCs migrated over shorter distances, but myelinated more rapidly and in higher proportions than did their fetal counterparts, with virtually no astrocytic coproduction. On an individual basis, each adult OPC–derived oligodendrocyte ensheathed and myelinated substantially more axons than did its fetal-derived counterparts (Fig. 4g).

Together, these observations suggest that isolates of human glial progenitor cells may provide effective cellular substrates for remyelinating the congenitally dysmyelinated or hypomyelinated brain. In practical terms, the choice of stage-defined cell type may be dictated by both the availability of donor material and the specific biology of the disease target. Their differences notwithstanding, fetal and adult-derived human OPC isolates were capable of achieving widespread and efficient myelination of the dysmyelinated brain, suggesting new strategies for the treatment of the congenital leukodystrophies and myelin disorders.

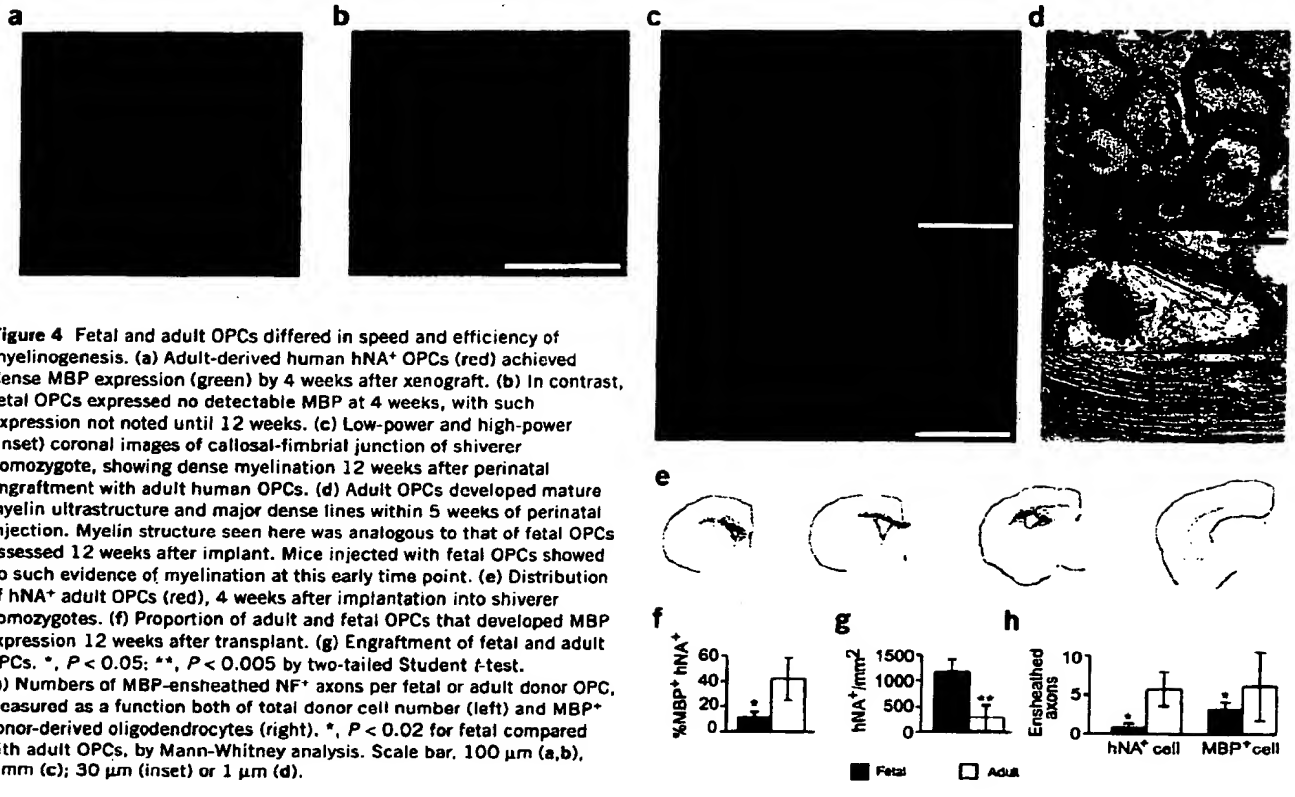

Figure 4 Fetal and adult OPCs differed in speed and efficiency of myelinogenesis. (a) Adult-derived human hNA$^+$ OPCs (red) achieved dense MBP expression (green) by 4 weeks after xenograft. (b) In contrast, fetal OPCs expressed no detectable MBP at 4 weeks, with such expression not noted until 12 weeks. (c) Low-power and high-power (inset) coronal images of callosal-fimbrial junction of shiverer homozygote, showing dense myelination 12 weeks after perinatal engraftment with adult human OPCs. (d) Adult OPCs developed mature myelin ultrastructure and major dense lines within 5 weeks of perinatal injection. Myelin structure seen here was analogous to that of fetal OPCs assessed 12 weeks after implant. Mice injected with fetal OPCs showed no such evidence of myelination at this early time point. (e) Distribution of hNA$^+$ adult OPCs (red), 4 weeks after implantation into shiverer homozygotes. (f) Proportion of adult and fetal OPCs that developed MBP expression 12 weeks after transplant. (g) Engraftment of fetal and adult OPCs. *, $P < 0.05$; **, $P < 0.005$ by two-tailed Student t-test. (h) Numbers of MBP-ensheathed NF$^+$ axons per fetal or adult donor OPC, measured as a function both of total donor cell number (left) and MBP$^+$ donor-derived oligodendrocytes (right). *, $P < 0.02$ for fetal compared with adult OPCs, by Mann-Whitney analysis. Scale bar, 100 μm (a,b), 1 mm (c); 30 μm (inset) or 1 μm (d).

METHODS

Cells. Fetal OPCs were extracted from 21- to 23-week-old human fetuses obtained at abortion. The forebrain ventricular and subventricular zones were dissected free and chilled on ice. The minced samples were dissociated using papain and DNase as described[20,21], always within 3 h of extraction, and maintained overnight in DMEM/F12/N1 with 20 ng/ml fibroblast growth factor. Adult-derived OPCs were collected from subcortical white matter samples obtained at surgery, as described[12,13]. The eight adult tissue samples used were derived largely from patients undergoing temporal lobe resection for medication-refractory epilepsy. No tissues were accepted from patients with known neoplastic disease. Both fetal and adult samples were obtained with consent, using protocols approved by the institutional review boards of Cornell–New York Presbyterian Hospital, and the Albert Einstein College of Medicine and Jacobi Hospital.

Sorting. The day after dissociation, cells from fetal samples were incubated in a 1:1 ratio with monoclonal antibody A2B5 supernatant (clone 105, American Type Culture Collection) for 30 min, then washed and labeled with fluorophore- or microbead-tagged rat antibody to mouse IgM (Miltenyi Biotec). In some instances, two-channel FACS was used to define the proportions and homogeneity of A2B5- and PSA-NCAM-defined subpopulations, using a FACSVantage SE/Turbo (Becton Dickinson) as described[13,21]. For preparative sorting before transplantation, A2B5$^+$ cells were prepared by magnetic separation (Miltenyi Biotec) according to the manufacturer's protocol. The bound cells were eluted and incubated with mouse antibody to PSA-NCAM (1:25; PharMingen) for 30 min, then with phycoerythrin-tagged secondary antibody (1:200). The PSA-NCAM$^+$ population was then removed by FACS, leaving a highly enriched pool of A2B5$^+$PSA-NCAM$^-$ cells. This PSA-NCAM immunodepletion step was omitted for adult samples, which were sorted on the basis of A2B5 only[12,20]. After sorting, both fetal and adult cells were maintained for 1–7 d in DMEM/F12/N1 with 20 ng/ml basic fibroblast growth factor (20 ng/ml) until implantation.

Transplantation and tagging. Homozygous shiverer mice were bred in our colony. Within 1 d of birth, pups were cryoanesthetized for cell delivery. Donor cells (1×10^5) in 2 µl of HBSS were injected through a pulled glass pipette and inserted through the skull into the presumptive corpus callosum. Transplants were directed to the corpus callosum at a depth of 1.0–1.2 mm, depending on the weight of the pup, which varied from 1.0 to 1.5 g. Pups were killed 4, 8, 12 or 16 weeks thereafter. For some experiments, recipient mice were injected for with bromodeoxyuridine (BrdU; 100 µg/g as a 1.5 mg/100 µl solution) every 12 hours 2 d before killing.

Immunohistochemistry. Transplanted cells were identified using antibody 1281 to human nuclei (Chemicon), monoclonal antibody 91 to cyclic nucleotide phosphodiesterase (CNP) protein (Sternberger and Meyer), rabbit antibody to S-100 (Sigma), rabbit antibody to human nestin (gift of H. Okano, Keio University), Sternberger monoclonal antibody 311 to NF, Sternberger monoclonal antibody 21 to human GFAP, rat antibody to BrdU (Harlan) and either Sternberger monoclonal antibody 94 to MBP or rat antibody 7349 to MBP (Abcam), all as described[7,12,20–22].

Confocal and electron microscopy. Confocal imaging was done using an Olympus Fluoview mated to an IX70 inverted microscope, as described[23]. Argon laser lines were used to achieve three-channel immunofluorescence detection of fluorescein-, Texas red– and Cy5-tagged antibodies; the latter was then pseudocolored blue for presentation. For confocal quantification of ensheathment efficacy, shiverer axons were scored as ensheathed when yellow index lines intersected NF$^+$ axon abutted on each side by MBP immunoreactivity. The proportion of ensheathed axons was defined as the incidence of MBP$^+$NF$^+$ axons divided by the total number of NF$^+$ axons in each field. For electron microscopy, animals were perfused and post-fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in 6% sucrose, then Vibratome-sectioned as alternating thick (400 µm) and thin (100 µm) sections. The latter were immunostained for MBP. Thick sections adjacent to thin sections with MBP expression were then processed in 1% osmium and 1.5% ferricyanide, stained with 1.5% uranyl acetate, embedded in Epon, cut as 100-nm thin sections onto Formvar-coated grids, stained with lead citrate and visualized using a JEOL100 electron microscope[24].

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

This work was supported by the National Multiple Sclerosis Society and National Institutes of Health/National Institute of Neurological Diseases and Stroke grants R01NS39559 and R01NS33106. We thank B. Poulos of the Albert Einstein College of Medicine tissue bank and S. Kelly of the American Biological Resource tissue bank for assistance with tissue acquisition, and H. Okano for the gift of antiserum to human nestin.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the Nature Medicine website for details).

Received 19 September; accepted 3 December 2003
Published online at http://www.nature.com/naturemedicine/

1. Lachapelle, F., Gumpel, M., Baulac, C. & Jacque, C. Transplantation of fragments of CNS into the brains of shiverer mutant mice: extensive myelination by transplanted oligodendrocytes. *Dev. Neurosci.* **6**, 326–334 (1983).
2. Roach, A., Takahashi, N., Pravtcheva, D., Ruddle, F. & Hood, L. Chromosomal mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in shiverer mutant mice. *Cell* **42**, 149–155 (1985).
3. Gansmuller, A. *et al.* Transplantations of newborn CNS fragments into the brain of shiverer mutant mice: extensive myelination by transplanted oligodendrocytes. II. Electron microscopic study. *Dev. Neurosci.* **8**, 197–207 (1986).
4. Gumpel, M. *et al.* Transplantation of human embryonic oligodendrocytes into shiverer brain. *Ann. NY Acad. Sci.* **495**, 71-85 (1987).
5. Gumpel, M., Gout, O., Lubetzki, C., Gansmuller, A. & Baumann, N. Myelination and remyelination in the central nervous system by transplanted oligodendrocytes using the shiverer model. Discussion on the remyelinating cell population in adult mammals. *Dev. Neurosci.* **11**, 132–139 (1989).
6. Seilhean, D., Gansmuller, A., Baron-Van Evercooren, A., Gumpel, M. & Lachapelle, F. Myelination by transplanted human and mouse central nervous system tissue after long-term cryopreservation. *Acta Neuropathol.* **91**, 82–88 (1996).
7. Mitome, M. *et al.* Towards the reconstruction of central nervous system white matter using neural precursor cells. *Brain* **124**, 2147–2161 (2001).
8. Yandava, B., Billinghurst, L. & Snyder, E. Global cell replacement is feasible via neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain. *Proc. Natl. Acad. Sci. USA* **96**, 7029–7034 (1999).
9. O'Leary, M.T. & Blakemore, W.F. Oligodendrocyte precursors survive poorly and do not migrate following transplantation into the normal adult central nervous system. *J. Neurosci. Res.* **48**, 159–167 (1997).
10. Learish, R.D., Brustle, O., Zhang, S.C. & Duncan, I.D. Intraventricular transplantation of oligodendrocyte progenitors into a fetal myelin mutant results in widespread formation of myelin. *Ann. Neurol.* **46**, 716–722 (1999).
11. Zhang, S.C., Ge, B. & Duncan, I.D. Adult brain retains the potential to generate oligodendroglial progenitors with extensive myelination capacity. *Proc. Natl. Acad. Sci. USA* **96**, 4089–4094 (1999).
12. Nunes, M.C. *et al.* Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. *Nat. Med.* **9**, 439–447 (2003).
13. Roy, N.S. *et al.* Identification, isolation, and promoter-defined separation of mitotic oligodendrocyte progenitor cells from the adult human subcortical white matter. *J. Neurosci.* **19**, 9986–9995 (1999).
14. Eisenbarth, G.S., Walsh, F.S. & Nirenberg, M. Monoclonal antibody to a plasma membrane antigen of neurons. *Proc. Natl. Acad. Sci. USA* **76**, 4913–4917 (1979).
15. Raff, M.C., Miller, R.H. & Noble, M. A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium. *Nature* **303**, 390–396 (1983).
16. Rao, M. & Mayer-Proschel, M. Glial-restricted precursors are derived from multipotential neuroepithelial stem cells. *Dev. Biol.* **188**, 48–63 (1997).
17. Piper, D. *et al.* Identification and characterization of neuronal precursors and their progeny from human fetal tissue. *J. Neurosci. Res.* **66**, 356–368 (2001).
18. Ridge, J., Fuchs, E. & Matzinger, P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science* **271**, 1723–1726 (1996).
19. Roser, B. Cellular mechanisms in neonatal and adult tolerance. *Immunol. Rev.* **107**, 179–202 (1989).
20. Windrem, M. *et al.* Progenitor cells derived from the adult human subcortical white matter disperse and differentiate as oligodendrocytes within demyelinated regions of the rat brain. *J. Neurosci. Res.* **69**, 966–975 (2002).
21. Keyoung, H.M. *et al.* Specific identification, selection and extraction of neural stem cells from the fetal human brain. *Nat. Biotechnol.* **19**, 843–850 (2001).
22. Louissaint, A., Rao, S., Leventhal, C. & Goldman, S.A. Coordinated interaction of angiogenesis and neurogenesis in the adult songbird brain. *Neuron* **34**, 945–960 (2002).
23. Benraiss, A., Chmielnicki, E., Lerner, K., Roh, D. & Goldman, S.A. Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. *J. Neurosci.* **21**, 6718–6731 (2001).
24. Goldman, S.A., Lemmon, V. & Chin, S.S. Migration of newly generated neurons upon ependymally derived radial guide cells in explant cultures of the adult songbird forebrain. *Glia* **8**, 150–160 (1993).
25. Franklin, R.J. & Blakemore, W.F. Transplanting oligodendrocyte progenitors into the adult CNS. *J. Anat.* **190**, 23–33 (1997).

CHAPTER

10

Progenitor Cells of the Adult Human Subcortical White Matter

Neeta S. Roy, Martha S. Windrem, and Steven A. Goldman

OLIGODENDROCYTE PROGENITOR CELLS OF THE ADULT MAMMALIAN BRAIN

Neural Progenitor Cells of the Adult Brain

Over the past few decades, historic notions of the structural immutability and cellular constancy of the adult vertebrate brain have been largely dispelled. Neurogenesis was first demonstrated in the rodent olfactory bulb and the hippocampus (Altman and Das, 1965, 1966; Kaplan and Hinds, 1977; Kaplan, 1985) and the songbird vocal control centers (Goldman and Nottebohm, 1983; Nottebohm, 1985). The phenomenon of adult neurogenesis has now been described throughout vertebrate phylogeny (Goldman, 1998), including monkeys (Gould *et al.*, 1998) and humans (Eriksson *et al.*, 1998; Kirschenbaum *et al.*, 1994; Pincus *et al.*, 1998). In all species yet examined, newly generated neurons seem to be generated from multipotential stem cells, the principal source of which appears to be the periventricular subependyma (SVZ) (Goldman *et al.*, 1993; Lois and Alvarez-Buylla, 1993). In addition, restricted pools of mitotically competent but phenotypically biased neuronal progenitor cells appear to derive from these stem cell populations. These neuronally restricted pools include the anterior subventricular zone of the forebrain and its rostral extension through the olfactory subependyma, as well as the subgranular zone of the hippocampus, each of which give rise almost exclusively to neurons *in vivo*. However, persistent multipotential stem cells have been reported in cultures derived from each of these regions (Gage *et al.*, 1998), suggesting that the apparent neuronal restriction of these progenitor populations may reflect not the inherent lineage capacity of the cells, so much as local environmental signals biasing toward neuronal differentiation (Seaberg and van der Kooy, 2002).

Besides these persistent neuronal progenitors and multipotential neural stem cells, more restricted lineages of glial progenitor cells also persist in the adult brain, in both the residual ventricular zone (Levison and Goldman, 1993; Luskin, 1993), as well as dispersed throughout the subcortical and cortical parenchyma (Gensert and Goldman, 1996; Levine *et al.*, 2001; Noble, 199 ; Reynolds and Hardy, 1997). Indeed, in contrast to the restricted distribution of neuronal progenitor cells and SVZ stem cells, oligodendrocyte progenitor cells (OPCs) seem to be extraordinarily widespread in the adult mammalian brain.

Oligodendrocyte Progenitors of the Normal Adult Rodent Brain

The principal class of OPCs in adult rodents is a bipotential astrocyte-oligodendrocyte progenitor cell designated the O-2A progenitor, by virtue of its generation *in vitro* of oligodendrocytes and type 2 astrocytes, the latter comprising the traditionally recognized fibrous astrocytes of the white matter. These cells were initially isolated from the optic nerves of perinatal rats, as O-2A progenitors (Raff *et al.*, 1983b). In neonatal rats, OPCs are characterized by expression of the GD3 and GQ gangliosides, the latter recognized by the monoclonal antibody A2B5, which has been used to identify this cell population (Noble *et al.*, 1992). Though similar progenitors were long ago reported in the adult optic nerve (Vaughn, 1969), the isolation of adult OPCs, or O-2A^{Adult} progenitors, was only accomplished relatively recently in rodents (Ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989). These cells have since been isolated from the adult rat ventricular zone, spinal cord, cerebellum, and subcortical white matter (Engel and Wolswijk, 1996; Gensert and Goldman, 1996; Levine *et al.*, 1993).

Antigenic Recognition of Adult OPCs

Little is known about the natural history of the adult OPC in normal adults. In histological sections of the adult rodent brain, OPCs have mainly been identified by their expression of both NG2 chondroitin sulfate proteoglycan (Levine *et al.*, 1993; Nishiyama *et al.*, 1997) and the platelet derived growth factor-alpha receptor (PDGF-αR). The expression of PDGF-αR and the NG2 epitope substantially overlaps in rats (Nishiyama *et al.*, 1996; Pringle *et al.*, 1992). Moreover, a persistent population of O4/NG2 co-expressing cells has been demonstrated in the adult rat cerebral cortex, effectively bridging the antigenic gap between early and committed OPCs (Reynolds and Hardy, 1997). On the basis of these studies, NG2-immunoreactivity has been developed as a surrogate marker for parenchymal oligodendrocyte progenitor cells. In addition, adult-derived OPCs have several features that may allow them to be distinguished: Whereas the perinatal OPC utilizes vimentin as an intemediate filament and does not express the oligodendrocytic sulfatide recognized by Mab O4, its adult counterpart does not express vimentin, but does express O4 (Shi *et al.*, 1998; Wolswijk and Noble, 1989; Wolswijk *et al.*, 1991). These parenchymal OPCs are present in both gray and white matter, and exist *in vivo* as extensively branched cells. The NG2 population represents as many as 5–8% of all the cells in the adult rodent brain (Dawson *et al.*, 2000); this is congruent with earlier estimates that 5% of all glia in the optic nerve may be progenitors (Vaughn and Peters, 1968).

Turnover

OPCs in the adult brain may include both slowly dividing cells in normal parenchyma and a quiescent cell population that responds only to injury or demyelination. *In vivo* studies of the adult cerebellar cortex reveal the presence of slowly dividing OPCs with a mitotic index of 0.2 to 0.3% (Levine *et al.*, 1993). Nevertheless, OPCs seem to constitute the main cycling population of the adult brain parenchyma. Bromodeoxyuridine (BrdU) labeling of the intact spinal cords of 13- to 14-week-old rats has shown that 10% of all cells in the white matter incorporated BrdU, of which 70% expressed NG2. In animals maintained for 4 weeks after BrdU injection, BrdU-labeled astrocytes and oligodendrocytes were noted, indicating that the cycling NG2 cells would have generated both cell types (Horner *et al.*, 2000). In studies using retroviral labeling to mark dividing cells, 35% of the cycling cells in the adult cortex co-labeled with NG2, and these were distinctly present as clusters. Furthermore, these NG2-positive clusters doubled in size every 3 months (Levison *et al.*, 1999). Using similar retroviral labeling techniques, the presence of cycling cells that preferentially give rise to oligodendrocytes has been shown in both the subventricular zone (SVZ) and subcortical white matter of adult rats (Gensert and Goldman, 1996; Levison and Goldman, 1993).

Lineage Potential

Previous studies had concluded that the perinatal OPC has a limited life span *in vivo*, which was attributed to a pattern of "exhaustive" symmetrical division and differentiation in

oligodendrocytes (Temple and Raff, 1986). Yet OPCs now appear to be maintained throughout life. This suggests that at least a fraction of OPCs may arise through a self-renewing, asymmetrical divisions, such that OPCs generate both differentiated progeny and themselves (Wren *et al.*, 1992). Indeed, adult OPCs of both rodents and humans retain their ability to generate oligodendrocytes and astrocytes over several generations *in vitro* (Tang *et al.*, 2000).

It seems likely that perinatal OPCs are the source of adult OPCs. Using time-lapse microcinematography, it has been shown that "founder cells" exhibiting properties of perinatal OPCs eventually give rise to cells with the properties of adult OPCs (Wren *et al.*, 1992). As noted, just as repetitive passage of perinatal OPCs gives rise to cells with adult OPC-like properties (Wolswijk *et al.*, 1990), slowly dividing adult OPCs can respond to FGF and PDGF by assuming the more rapid expansion kinetics typical of perinatal OPCs. Together, these data argue that perinatal and adult OPCs constitute two points along the differentiation spectrum of a common lineage. Nonetheless, diversification within that lineage may nonetheless have resulted in substantial phenotypic heterogeneity among adult OPCs (Gensert and Goldman, 2001).

Humoral Control of Oligoneogenesis

Adult and perinatal OPCs share many commonalities in their responses to humoral growth factors, but nonetheless exhibit differential responses to both neural mitogens and differentiation agents. These include, but are by no means limited to, the following:

1. Platelet derived growth factor. PDGF is perhaps the most prominent described oligotrophin and has been implicated in both the mitotic expansion of OPCs and their initiation of terminal lineage commitment (Hart *et al.*, 1989a; Noble *et al.*, 1988; Raff *et al.*, 1988; Wolswijk *et al.*, 1991). OPCs uniquely express high levels of PDGFα receptor, and can be specifically identified on that basis (Ellison and de Vellis, 1994; Fruttiger *et al.*, 1999; Hart *et al.*, 1989b). In response to PDGF, both perinatal and adult OPCs enter the mitotic cycle. However, cycling time differs in the two cell populations, in that adult OPCs have a slow, 3- to 4-day cell cycle, whereas perinatal OPCs divide daily (Noble *et al.*, 1988); (Wolswijk *et al.*, 1991). In OPCs derived from the adult spinal cord, PDGF alone supports the slow mitotic expansion of OPCs, as the cells divide slowly and undergo asymmetrical division, generating a differentiated oligodendrocyte and another progenitor (Engel and Wolswijk, 1996). However, in the presence of PDGF and FGF, adult OPCs accelerate their cycle progression, dividing rapidly and apparently symmetrically to yield additional progenitors. They then assume the bipolar morphology and A2B5 immunoreactivity of oligodendrocyte progenitor cells, but fail to generate oligodendrocytes without downstream inductive differentiation. As a corollary to this "perinatalization" of adult-derived OPCs, cultures of perinatal OPCs expanded over long periods of time in the presence of PDGF alone develop the cyclicity of adult OPCs (Tang *et al.*, 2000). These results suggest that in rodents at least, perinatal and adult-derived OPCs represent points on a continuum of differentiative state, rather than discrete phenotypes

2. Fibroblast growth factor. FGF differentially regulates OPC proliferation and differentiation in culture and modulates gene expression of its own receptors in a developmental and receptor type-specific manner (Bansal *et al.*, 1996). Most *in vitro* studies show that bFGF is a major mitogen for cells in the oligodendrocyte lineage (Besnard *et al.*, 1989; Eisenbarth *et al.*, 1979). It has been shown to stimulate the proliferation of late progenitors and inhibit their terminal differentiation (Bansal and Pfeiffer, 1994; McKinnon *et al.*, 1990). More important, it establishes the responsiveness to PDGF by up-regulating the expression of PDGF-αR (McKinnon *et al.*, 1990). Most studies with adult OPCs show that bFGF is most mitogenic when used in combination with PDGF (Mason and Goldman, 2002; Tang *et al.*, 2000). Recently it has been shown that OPCs maintained in the presence of bFGF eventually become resistant to replicative senescence (Tang *et al.*, 2001). Besides its well-documented effect on OPCs, bFGF also induces the down-regulation of myelin genes, such as myelin basic protein (MBP), in mature oligodendrocytes without reverting

them to the progenitor phenotype or effecting reentry into the cell cycle (Bansal and Pfeiffer, 1997; Grinspan *et al.*, 1993).

3. Neurotrophin-3 (NT3). Whether NT3 has proliferative or differentiative effect on OPCs is yet unresolved. One study indicated that NT3, specifically in combination with PDGF, is proliferative for post-natal OPCs both *in vitro* and *in vivo* (Barres *et al.*, 1994b). Other studies, however, found that NT3 is not proliferative for adult OPCs alone or in combination with PDGF and bFGF (Engel and Wolswijk, 1996; Ibarrola *et al.*, 1996). Perhaps this differential response may be a function of the different OPC-types that have been used for the two studies. In the contused adult spinal cord, NT3 has been shown to increase OPC proliferation and myelination (McTigue *et al.*, 1998). A recent *in vitro* study with OPCs from adult spinal cord dissociates indicates that NT3 induced myelination and the proliferation of O4+/O1-cells (Yan and Wood, 2000).

4. Neuregulin. The neuregulins are a family of soluble and transmembrane protein isoforms, of which glial growth factor 2 (GGF2) is a member (Adlkofer, 2000). The neuregulins act upon erbB receptors, in particular on the erbB2, 3, and 4 heterodimeric receptors (Buonanno and Fischbach, 2001). Perinatal OPCs divide in response to GGF provided cAMP levels are high, so that adenyl cyclasae and erbB stimulation may operate synergistically as glial progenitor mitogens (Shi *et al.*, 1998). Canoll *et al.* observed a similar proliferative effect on $O4^+/O1^-$ progenitors (Canoll *et al.*, 1996). Adult OPCs respond to GGF2 as well, although their mitogenic activation by GGF2 appears to require the concurrent activation of the PDGF receptor, along with elevated cAMP. An interesting feature of neuregulins includes their induction of phenotypic reversion by differentiated oligodendrocytes (Canoll *et al.*, 1999). OPCs produce neuregulins (Raabe *et al.*, 1997) as well as respond to it (Shi *et al.*, 1998). Since they express full-length neuregulin erbB receptors, OPCs may utilize neuregulins as an autocrine factor, as well as a neuronally derived oligotrophin (Fernandez *et al.*, 2000). This is likely to obtain in the environment of the adult human white matter, from which oligodendrocytes have similarly been shown to produce neuregulins and express receptors to them (Cannella *et al.*, 1999; Deadwyler *et al.*, 2000).

5. Triiodothyronine. When OPCs derived from optic nerves or cerebral hemispheres are cultured in the presence of T3, they immediately stop dividing and differentiate into oligodendrocytes. In fact, the number of times an OPC can divide varies inversely with its concentration of T3, implicating T3 as an oligodendrocytic differentiation factor (Baas *et al.*, 1997). T3 seems to play a major role in controlling the timing of OPC differentiation (Barres *et al.*, 1994a). Accordingly, hypothyroid states have been associated with deficits in early myelination in neonatal cretinism, which may reflect a failure in T3-mediated OPC expansion.

6. Insulin growth factor-1 (IGF-1). During development, high levels of IGF1 are observed just before active myelination commences (Bach *et al.*, 1991; Carson *et al.*, 1993). IGF-1 increases proliferation and survival, enhance differentiation, and modulate the expression of MBP in both OPCs and oligodendrocytes (Barres *et al.*, 1992; McMorris and Dubois-Dalcq, 1988; Saneto *et al.*, 1988).

Oligodendrocyte Progenitors of the Adult Human Brain

The earliest evidence that the adult human brain harbors oligodendrocyte progenitors came from early studies of MS lesions. Histopathologically, these lesions were found to harbor regions of extensively remyelinated axons, as well as numerous free oligodendrocytes (Moore *et al.*, 1985; Prineas and Connell, 1979; Prineas *et al.*, 1984). Subsequent studies identified populations of immature cells expressing the neural carbohydrate epitope HNK1; these were postulated to comprise early oligodendroglia, although these early studies were unable to identify any definitive oligodendrocyte progenitor cell phenotype (Prineas *et al.*, 1989; Wu and Raine, 1992).

PDGF-αR expressing OPCs have been shown in both MS lesions and surrounding normal white matter (Scolding *et al.*, 1998). These PDGF-αR^+ cells were found to be more

frequent in or near MS lesions compared to normal surrounding white matter (WM), and those near lesions were more often cycling, as revealed by immunoreactivity for Ki67, a marker of proliferation (Maeda *et al.*, 2001). Corroborating these observations with another marker of phenotype, the NG2 chondroitin sulfate proteoglycan was demonstrated in both normal adult human WM and MS lesions. As in their rodent counterparts, human $NG2^+$ cells were found to be extensively ramified. Cells morphologically similar to $NG2^+$ cells were reported to express PDGF-αR as well, although co-expression of the two by a common phenotype has yet to be directly demonstrated.

Premyelinating oligodendrocytes—defined by their expression of proteolipid protein (PLP), and their contiguity with axons despite an absence of attendant ensheathment—have also been shown in such MS lesions (Chang *et al.*, 2002). Interestingly, $NG2^+$ cells are virtually absent from lesions lacking premyelinating oligodendrocytes. This suggests that $NG2^+$ cells might be the source of these premyelinating oligodendrocytes. However, the NG2 chondroitin sulfate may not be specific to OPCs in the adult human brain, as microglial cells express or sequester high levels of NG2-IR (Pouly *et al.*, 1999; also Nunes, Roy, and Goldman, unpublished observations). Indeed, in dissociates of both fetal and adult human brain tissue, most $NG2^+$ cells were microglial (Pouly *et al.*, 1999). To establish a more reliable marker of OPCs in adult human tissues, Scolding *et al.* thus assessed the phenotypic specificity of two cardinal markers of OPC phenotype in rodents, specifically the PDGF-α receptor and the A2B5 epitope represented by the GQ ganglioside. By scoring the incidence of both $PDGF\text{-}\alpha R^+$ and $A2B5^+$ cells in tissue print preparations of adult human white matter, Scolding and colleagues determined that these markers recognize a common parenchymal progenitor cell population. On this basis, they were able to report the first estimates of the incidence of oligodendrocyte progenitor cells in the human white matter (Scolding *et al.*, 1999).

Despite this wealth of histological assessment of parenchymal progenitor cells, relatively few studies have yet correlated the antigenic expression patterns of single parenchymal phenotypes with their lineage potential, either *in vivo* or *in vitro*. As a result, it remains unclear if the expression of markers such as GD3, NG2, A2B5, or PDGF-αR is specific to adult OPCs, or whether it instead is shared among different, already discrete lineages at similarly early points in their phenotypic specification. The uncertain lineage potential of histologically antigen-defined oligodendrocyte progenitor cells has derived in part from an historic inability to identify or isolate these cells from human brain tissues. An early attempt to identify oligodendrocyte progenitors in dissociates of adult human brain (Kim *et al.*, 1983) was followed by successful *in vitro* and *in vivo* demonstrations of immature oligodendroglia, which were termed pro-oligodendrocytes because of their post-mitotic state. These cells were defined as being $O4^+/A2B5^-/GalC^-$ (Armstrong *et al.*, 1992). Pro-oligodendrocytes were further characterized and found to express the PDGF-αR in tissue, where they were estimated to constitute 2% of the total cell population (Gogate *et al.*, 1994). Subsequent studies of the adult human white matter *in vitro* revealed the presence of mitotic cells that could give rise to oligodendrocytes, though the identity of the precursor remained unclear (Roy *et al.*, 1999; Scolding *et al.*, 1995);.

Humoral Control of Adult Human Oligodendrocyte Progenitor Cells

Human and rodent OPCs differ not only in their antigenic expression patterns, as noted, but also as in their responses to humoral growth factors. Adult human OPCs do not proliferate in response to bFGF, PDGF, or IGF-1, each of which can act singly as a mitogen for rodent OPCs (Armstrong *et al.*, 1992; Gogate *et al.*, 1994; Prabhakar *et al.*, 1995). Instead, in human OPCs, IGF-1 has been shown to increase the proportion of post-mitotic pro-oligodendrocytes and to promote the maturation of these cells as oligodendrocytes (Armstrong *et al.*, 1992). Human OPCs also seem to be mitotically unresponsive to astrocyte conditioned medium (Armstrong *et al.*, 1992; Gogate *et al.*, 1994; Prabhakar *et al.*, 1995; Scolding *et al.*, 1995). As noted previously, neuregulin supports the expansion of OPCs and is released by neurons in an activity-dependent manner that might allow the activity-dependent modulation of OPC expansion (Canoll *et al.*, 1996). However, these

observations have yet to be verified as operative in human OPCs. Indeed, little data are available on the factor responsiveness of human OPCs, despite the overt clinical importance of establishing the optimal expansion and differentiation conditions for these cells. Rather, the study of their growth factor responsiveness, patterns of receptor expression, and likely paracrine interactions with other parenchymal cell populations have been impeded by the inability to identify and isolate OPCs from the adult human brain, and hence the lack of material for molecular and cellular analysis.

Isolation of Adult Human Oligodendrocyte Progenitor Cells

To address the need for isolating enriched populations of adult OPCs, we used promoter-specified fluorescent activated cell sorting (FACS) to identify and extract these cells from adult human brain tissue. Traditionally, FACS has been used to sort live cells on the basis of surface antigen expression, particularly in the hematopoetic system, in which FACS has been used to define and isolate the major stem cell and intermediate progenitor phenotypes generated during lymphopoesis and hematopoiesis. However, the application of FACS to the nervous system was stymied by the lack of identified surface antigens specific to stage or phenotype among neural cells. Yet in 1994, the green fluorescence protein was first identified as a live cell reporter of gene expression (Chalfie *et al.*, 1994). By placing GFP under the transcriptional control of promoters regulating the expression of cell-specific genes, we were able target specific cell phenotypes for FACS isolation. We first applied this approach to extracting neuronal progenitor cells from the fetal ventricular zone (Wang *et al.*, 1998), by transducing ventricular zone cells with GFP placed under the control of the Tα1 tubulin promoter, an early neuronal regulatory sequence (Gloster *et al.*, 1994; Miller *et al.*, 1987, 1989). This approach has since allowed us to isolate neuronal progenitor cells from both the adult human ventricular zone (VZ) and hippocampus (Roy *et al.*, 2000a, 2000b). In addition, by modifying our choice of promoters to those specifically active in even earlier neural progenitors, we were able to isolate less committed neural stem cells from both the adult and fetal human brain (Keyoung *et al.*, 2001; Roy *et al.*, 2000a, 2000b).

The development of promoter-based FACS gave use the means to identify and then isolate oligodendrocyte progenitor cells from the adult human brain (Fig. 10.1). To this end, we used the early promoter for an early oligodendrocyte protein, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (Scherer *et al.*, 1994; Vogel and Thompson, 1988). CNP protein is the earliest myelin-associated protein known to be expressed in developing oligodendrocytes. It is expressed by oligodendrocytes at all ontogenetic stages (Sprinkle, 1989; Weissbarth *et al.*, 1981), including by newly generated cells of oligodendrocytic lineage within the subventricular zone and their mitotic precursors (Scherer *et al.*, 1994; Yu *et al.*, 1994). The 5' regulatory region of the CNP gene includes two distinct promoters, P2 and P1, which encode for two CNP isoforms, CNP1 (46kDA) and CNP2 (48 kDa). These promoters are sequentially activated during development, with the more upstream P2 promoter (P/CNP2) directing transcription to immature oligodendrocytes and their progenitors (Gravel *et al.*, 1998; O'Neill *et al.*, 1997). On this basis, P/CNP2 was chosen to identify oligodendrocyte progenitors from adult human subcortical white matter (Roy *et al.*, 1999). P/CNP2:hGFP was transfected into dissociate of adult human white matter, and following GFP expression 3 to 4 days later, the P/CNP2:GFP$^+$ cells were isolated by FACS (Roy *et al.*, 1999). These cells, maintained in serum-deficient media supplemented with FGF2, PDGF, and NT-3, were bipolar, immunoreactive for A2B5, incorporated BrdU from their culture media, and developed into O4$^+$ oligodendrocytic *in vitro* (Fig. 10.2). These data indicated that the P/CNP2:hGFP-defined cells were mitotic oligodendrocyte progenitors. On this basis, P/CNP2:hGFP$^+$ oligodendrocyte progenitors were extracted directly from adult human WM dissociates using FACS. We found that an average of 0.5 $\pm$ 0.1% of all white matter cells directed P/CNP2:hGFP expression. Given a transfection efficiency of 13.5%, determined using the percentage of GFP expressing cells obtained with p/CMV:GFP for noncell type specific transfection, it could be estimated that over 4% of adult human subcortical WM are P/CNP2-defined progenitors. Immediately after FACS, these P/CNP2:hGFP-separated cells were initially bipolar, and

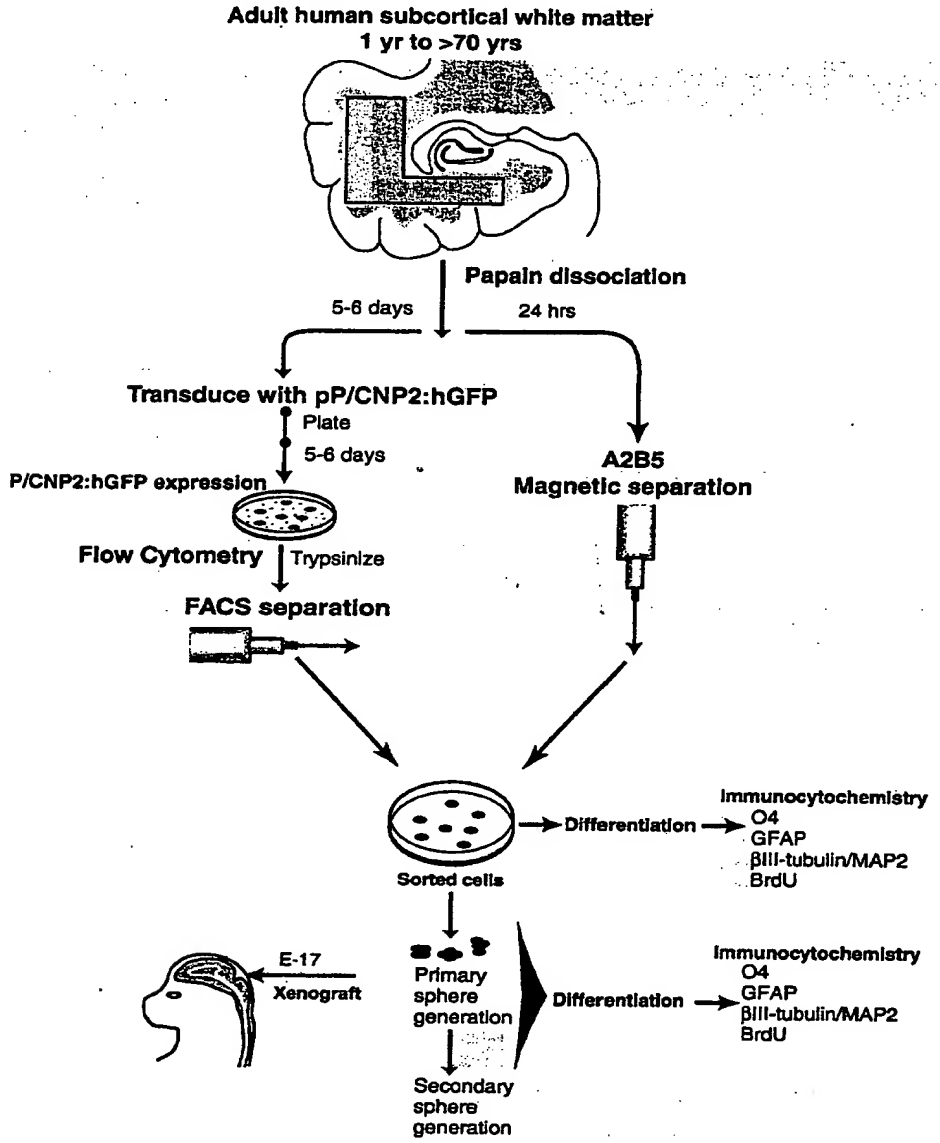

FIGURE 10.1
Oligodendrocyte progenitor cells may be specifically targeted and isolated from the white matter. This schematic outlines basic strategies for isolating oligodendrocyte progenitor cells from the adult white matter, using either fluorescence-activated cell sorting (FACS) or a higher-yield, less specific alternative immunomagnetic isolation (MACS).

expressed the early oligodendrocytic marker A2B5, but none of the differentiated markers O4, O1, or galactocerebroside; over half incorporated BrdU. When followed up to a month in culture, >80% of the PCNP2:hGFP$^+$ cells become oligodendrocytes, progressing through a succession of A2B5, O4, and galactocerebroside expression, recapitulating the developmental sequence of antigenic expression (Noble, 1997). Thus, with this strategy not only was the existence of oligodendrocyte progenitors established in adult human white matter, but a method was developed to separate the progenitors in a form appropriate for engraftment and further analysis.

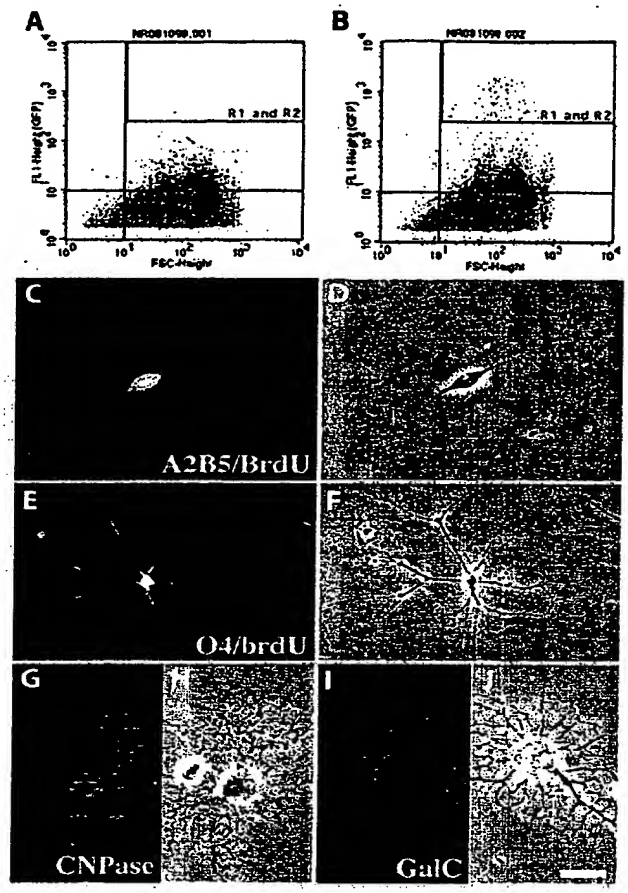

FIGURE 10.2
Sorted human white matter progenitor cells typically mature as oligodendrocytes. (A–B) A representative sort of a human white matter sample, derived from the frontal lobe of a 42-year-old woman during repair of an intracranial aneurysm. This plot shows 50,000 cells (sorting events) with their GFP fluorescence intensity plotted against forward scatter (a measure of cell size). Part A indicates the sort obtained from a nonfluorescent P/hCNP2:*lacZ*-transfected control, while part B indicates the corresponding result from a matched culture transfected with P/hCNP2:hGFP. (C–D) A bipolar $A2B5^+/BrdU^+$ cell, 48 hours after FACS. (E–F) By 3 weeks post-FACS, P/CNP2:hGFP-sorted cells developed multipolar morphologies and expressed oligodendrocytic O4 (red). These cells often incorporated BrdU, indicating their *in vitro* origin from replicating $A2B5^+$ cells. (G–I) Matched phase (G, I) and immunofluorescent (H, J) images of maturing oligodendrocytes, 4 weeks after P/CNP2:hGFP-based FACS. These cells expressed both CNP protein (H) and galactocerebroside (J), indicating their maturation as oligodendrocytes. Scale bar = 20 μm. Taken from Roy *et al.*, 1999; with permission.

Antigenicity of Oligodendrocyte Precursor Cells

As described earlier, virtually all P/CNP2:hGP-defined OPCs are immunoreactive for A2B5 (Roy *et al.*, 1999). This permitted us to use A2B5-based sorting to increase the yield of isolated progenitors, to numbers sufficient for experimental transplantation. Although both immature neurons and glia express A2B5-immunoreactivity during development (Aloisi *et al.*, 1992; Eisenbarth *et al.*, 1979; Lee *et al.*, 1992), the adult subcortical parenchyma is relatively devoid of young neurons, allowing A2B5 to be used as a selective marker of glial and oligodendrocyte progenitor cells (Raff *et al.*, 1983a; Satoh *et al.*, 1996; Scolding *et al.*, 1999). The specific use of A2B5 as an antigenic surrogate for P/CNP2:hGFP-defined OPCs has thus constituted a significant practical advance. By extracting OPCs via A2B5-based surface-antigen based sorting, the limitations of transfection-based tagging, which include

direct cytotoxicity as well as low efficiency, can be avoided entirely. As a result, the practical issue of acquiring sufficient numbers of viable OPCs to permit transcriptional and biochemical analysis, as well as engraftment studies, can now be effectively addressed.

Multipotential Progenitors of the Adult Human White Matter

Like their lower species counterparts, human OPCs may not be strictly dedicated or autonomously programmed to oligodendrocytic differentiation. When purified from adult human subcortical tissue, derived from surgically resected temporal lobe, white matter progenitor cells (WMPCs) give rise largely to oligodendrocytes. However, when grown under conditions of very low density, we noted that these cells also generate occasional neurons (Roy *et al.*, 1999). On this basis, we asked whether the white matter progenitor cells of the adult human brain might actually constitute a type of multipotential neural progenitor or neural stem cell. We found that white matter progenitor cells, purified by FACS from the adult human brain, can indeed generate neurons as well as both major glial cell types—astrocytes and oligodendrocytes—when raised in culture under conditions of high purity and low density (Nunes *et al.*, 2003). Under these conditions, the cells are effectively removed from other cells, as well as from the proteins that other cells may secrete. Under these conditions, the sorted progenitor cells divide and expand as multipotential clones that generate neurons as readily as oligodendrocytes (Fig. 10.3). They can continue to divide and expand for several months in culture, dividing to increase their

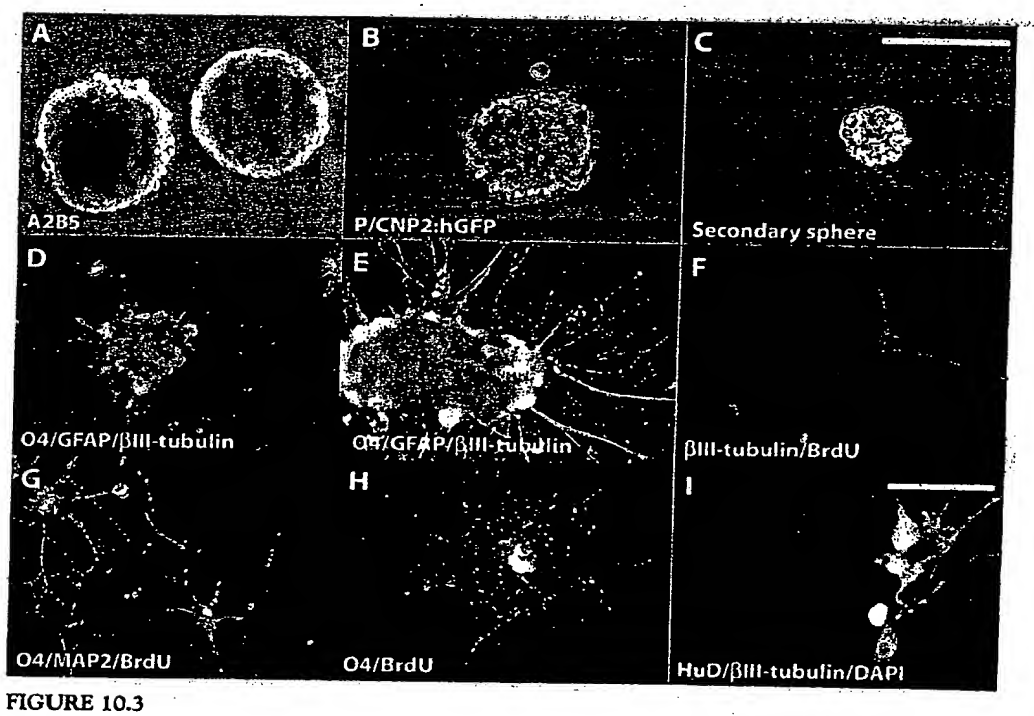

FIGURE 10.3
Adult human WMPCs give rise to multipotential neurospheres. (A) First-passage spheres generated from A2B5-sorted cells 2 weeks post-sort. (B) First-passage spheres arising from P/CNP2:hGFP sorted cells, 2 weeks. (C) Second-passage sphere derived from an A2B5-sorted sample, at 3 weeks. (D) Once plated onto substrate, the primary spheres differentiated as βIII-tubulin$^+$ neurons (red), GFAP$^+$ astrocytes (blue), and O4$^+$ oligodendrocytes (green). (E) Neurons (red), astrocytes (blue), and oligodendrocytes (green) similarly arose from spheres derived from P/CNP2:GFP-sorted WMPCs. (F–H) BrdU incorporation (blue) revealed that new neurons (F: βIII-tubulin in red; G: MAP2 in red) and oligodendrocytes (H: O4 in green) were both generated *in vitro*. (I) βIII-tubulin$^+$ neurons (green) co-expressed neuronal Hu protein (Barami *et al.*, 1995; Marusich *et al.*, 1994) (red, yielding; yellow double-label). Nuclei counterstained with DAPI (blue). From Nunes *et al.* (2003). Scale: A–E, 100 μm; F–I, 24 μm.

numbers in the process. Moreover, upon xenograft to the developing fetal rat forebrain, adult human WMPCs can mature into neurons as well as oligodendrocytes and astrocytes *in vivo*, in a region- and context-dependent manner (Fig. 10.4). The nominally glial progenitor cell of the adult human white matter thus appears to constitute a multipotential neural progenitor. These cells appear to be typically restricted by their local brain environment to produce only oligodendrocytes and some astrocytes, in response to local environmental signals whose identities remain to be established. But when removed from the

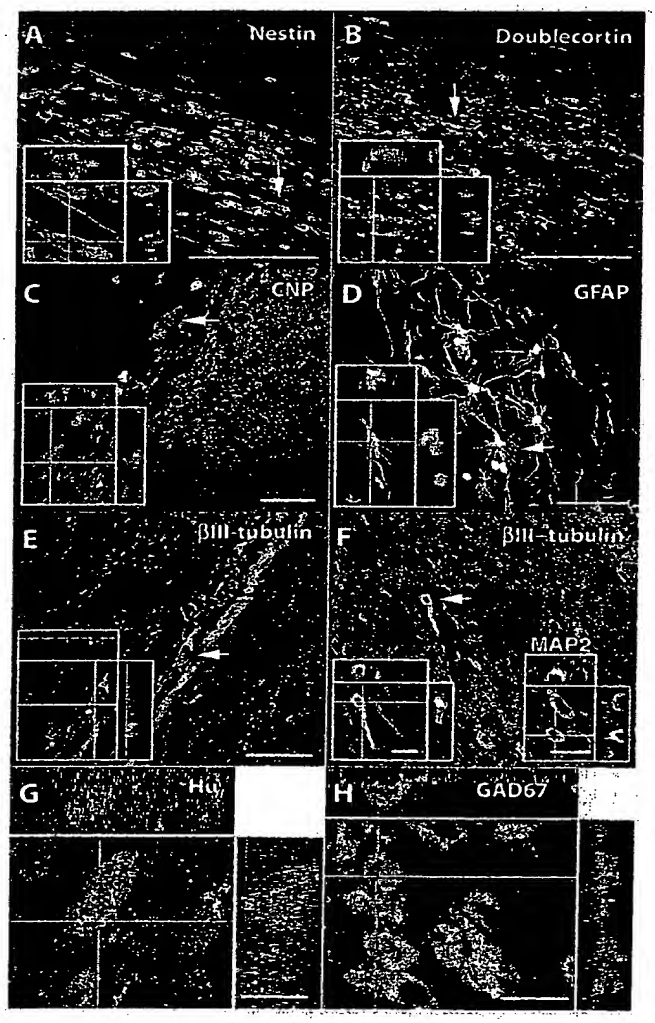

FIGURE 10.4

WMPCs engrafted into fetal rats gave rise to neurons and glia in a site-specific manner. Sections from a rat brain implanted at E17 with A2B5-sorted WMPCs and sacrificed a month after birth. These cells were maintained in culture for 10 days prior to implant. (A–B) Nestin$^+$ (red) progenitors and doublecortin$^+$ (red) migrants, respectively, each co-expressing human nuclear antigen (hNA, green) in the hippocampal alvius. (C) CNP$^+$ oligodendrocytes (red) that were found exclusively in the corpus callosum. (D) A low-power image of GFAP$^+$ (green, stained with anti-human GFAP) astrocytes along the ventricular wall. (E) βIII-tubulin$^+$ (green)/hNA$^+$ (red) neurons migrating in a chain in the hippocampal alvius. (F) βIII-tubulin$^+$ and MAP2$^+$ (inset in part F) neurons in the striatum, adjacent to the RMS (antigens in green; hNA in red; yellow: double-stained human nuclei). (G) An Hu$^+$/hNA$^+$ neuron in the septum. (H) An hNA$^+$ (green)/GAD-67$^+$ (red) striatal neuron. Insets in each figure show orthogonal projections of a high-power confocal image of the identified cell (arrow). From Nunes *et al.* (2003). Scale: A–E, 40 μm; F–H, 20 μm.

environment of the brain and from other brain cells, these cells proceed to make all brain cell types, including neurons and glia, and remain able to do so for long periods of time in culture.

This observation has precedent in lower species. Progenitor cells capable of giving rise to multiple lineages, including oligodendrocytes and neurons, have been consistently derived from the cortical and subcortical parenchyma as well as from the ventricular zone of embryos (Davis and Temple, 1994; Qian *et al.*, 1997; Williams *et al.*, 1991). Similar multipotential progenitors have shown to exist in early postnatal rat cortex (Marmur *et al.*, 1998). A more recent study suggested that postnatal rat optic nerve derived O-2A progenitor cells could be "reprogrammed" to multipotential stem cells capable of generating neurons (Kondo and Raff, 2000). This was achieved by sequential exposure of O-2A progenitors to serum to induce astrocytic differentiation, followed by their expansion in the presence of bFGF in serum-free conditions. Constant mitogenic stimulation of adult rat forebrain parenchymal cells with FGF2 has been shown to result in the generation of neurons as well as astrocytes and oligodendrocytes (Palmer *et al.*, 1995; Richards *et al.*, 1992). Together, these observations of the multilineage potential of CNS glial progenitors suggest that the apparent lineage commitment of progenitors might depend on epigenetic factors. As a result, the nominally glial progenitors of the adult white matter may retain far more lineage plasticity and competence than traditionally appreciated. Adult subcortical P/CNP2:hGFP$^+$ progenitors, though competent to generate multiple cell types, may therefore be restricted to the oligodendrocytic lineage by virtue of the epigenetic bias imparted by their environment before their isolation.

A corollary of the environmental restriction of WMPC phenotype is that other, non-white-matter-derived neural progenitors might similarly restrict to oligodendrocytic lineage when presented to the environment of the adult white matter. Indeed, several groups have reported that EGF-expanded murine neural stem cells differentiate as oligodendrocytes upon xenograft (Mitome *et al.*, 2001); remarkably, in none of these models were substantial numbers of oligodendrocytes generated *in vitro*. Similarly, v-myc transformed neural stem cells transplanted to perinatal mice can differentiate as oligodendrocytes once recruited to the white matter (Yandava *et al.*, 1999), but not otherwise, and never *in vitro*.

The Distribution and Heterogeneity of White Matter Progenitor Cells

The persistence and sheer abundance of WMPCs in the adult human brain is striking: Over 3% of the white matter cell population may be sorted on the basis of CNP2:GFP-based FACS, and over half of these cells are mitotically active upon isolation (Roy *et al.*, 1999). That being said, the extent to which this parenchymal progenitor cell population is homogeneous remains unclear; by limiting dilution analysis, only 0.2% of its cells are multipotential (Nunes *et al.*, 2003). Nonetheless, the very existence of multipotential progenitors scattered throughout the white matter parenchyma forces us to reconsider our understanding of both the nature and incidence of neural stem cells in the adult brain and challenges our conception of the supposed rarity of adult neural progenitor and stem cells. In doing so, they point to an abundant and widespread source of cells, which may be used both as a target for pharmacological induction and as a cell type appropriate for therapeutic engraftment to the diseased adult brain.

THERAPEUTIC POTENTIAL OF HUMAN OLIGODENDROCYTE PROGENITOR CELLS

The Natural History of Remyelination in the Adult CNS

The existence of active remyelination in the adult human brain has been mainly derived from observations of MS lesions. However, it has been unclear whether that remyelination has been the result of local expansion of parenchymal OPCs or of the recruitment of distant OPCs to sites of acute demyelination. Moreover, the source and in resting phenotype of the

remyelinating cells has been unclear. To address these questions, Gensert and Goldman (1997) used a combination of retroviral labeling and lysolecithin-induced demyelination to show that normally cycling cells of the adult rodent WM can differentiate as myelinating oligodendrocytes (Gensert and Goldman, 1997). Interestingly, before the endogenous OPCs participated in remyelination, they proliferated locally. Similarly, mice infected with a demyelinating murine hepatitis virus exhibited almost a 14-fold increase in PDGF-αR^+ OPCs in the lesion bed (Redwine and Armstrong, 1998). Other studies using rats with EAE or ethidium bromide lesions have shown that after remyelination, OPC numbers were stable (Levine and Reynolds, 1999). This in turn suggested that OPCs can undergo asymmetric division to replicate themselves while generating a differentiating oligodendrocyte.

There appears to be limited survival of OPCs in demyelinated lesions; as a result, most remyelination may be accomplished by unaffected OPCs recruited from the lesion surround. Carroll *et al.* have shown that OPCs in regions adjacent to immunolytic lesions first respond by dividing, followed by their migration into the lesion, and ultimate myelinogenesis (Carroll *et al.*, 1998). Similar observations were made in the demyelinated adult spinal cord, where the population of $NG2^+$ cells expanded significantly in areas adjacent to demyelinating lesions. In this case though, the proliferating pool appeared unable to sustain its self-renewal, as $NG2^+$ cells were depleted following remyelination (Keirstead *et al.*, 1998). Using X-irradiation, Chari and Blakemore (2002) reported that locally recruited $NG2^+$ and PDGF-α R^+ OPCs can repopulate depleted areas over distances of approximately 0.5 mm per week in the first month. No secondary progenitor loss was observed in those surround regions from which progenitor cells were recruited, indicating dynamic replacement of the emigrants (Chari and Blakemore, 2002). However, the question of how far the progenitor population can migrate in intact tissue remains debatable, an issue of particular concern for remyelination strategies involving transplantation (Franklin and Blakemore, 1997). Complicating matters further, recent studies have reported an age-related decrease both in recruitment of OPCs and in their subsequent differentiation (Sim *et al.*, 2002).

Candidate Cellular Vectors for Experimental Remyelination via Progenitor Implantation

Progenitor cells capable of local cell genesis therefore persist throughout the subcortical white matter of the adult brain, where they might constitute a potential substrate for cellular replacement and local repair. However, several criteria must be considered when evaluated the transplantation potential of any progenitors. These include the ability of transplanted cells to survive in the host environment, to migrate accurately to the target lesion or tissue type, to generate myelin, to ensheath host axons, and to achieve a degree of myelination capable of functional reconstitution. To assess the myelinogenic potential of transplanted cells, a variety of cell types including multipotential stem cells and OPCs, derived from both animals and humans, have been tested in both developmentally dysmyelinated and experimentally demyelinated models of myelin loss.

Neural Stem Cells and Progenitors from the Fetal Brain

Human fetal brain cells have been found to have robust myelinogenic capacity in the *shiverer* mice (Gansmuller *et al.*, 1986; Gumpel *et al.*, 1987, 1989). Cells isolated from the rodent or human fetal forebrains at various gestational ages, and expanded *in vitro* under a variety of serum-free, factor-supplemented conditions, have been used as sources of transplantable cells (Ader *et al.*, 2001; Brustle *et al.*, 1998; Carpenter *et al.*, 1999; Fricker *et al.*, 1999; Hammang *et al.*, 1997). However, there are potential risks to prolonged *in vitro* expansion, since the cells are not only exposed to exogenous mitogens, but also to autocrine factors in artificially high concentration, and to paracrine agents produced by the neurons and glia present within the initially mixed cultures. As a result, propagated stem or progenitor cells may not retain or reflect the lineage potential or differentiation competence of the native progenitor cells from which they derived. Two recent studies have highlighted the effects of *in vitro* expansion of cells prior to transplant. Buchet *et al.* observed that freshly isolated cells

proliferated longer and gave rise to very extended grafts before differentiating into neurons and glia while cells that were expanded prior to transplant showed poor proliferation and quick differentiated capacity (Buchet *et al.*, 2002). In contrast, Englund *et al.* found that after 9 weeks of expansion, human fetal brain cells lost the capacity to differentiate and remained as undifferentiated progenitors when transplanted into adult recipients (Englund *et al.*, 2002). To circumvent the issue of paracrine effects on defined stem cells in mixed culture, several groups have developed methods of directly isolating neural stem cells from tissue, thereby preventing their *in vitro* exposure to differentiated cell products during either isolation or expansion (Keyoung *et al.*, 2001; Uchida *et al.*, 2000).

Neural Stem Cells and Progenitors from Adult Brain

Several studies describe the use of neural stem cells derived primarily from the adult rat and human VZ, and then propagated as neurospheres, as a potential source of myelinogenic cells (Akiyama *et al.*, 2001; Kukekov *et al.*, 1999; Zhang *et al.*, 1999). As described earlier, the adult human white matter harbors an abudance of oligodendrocyte progenitors. By virtue of their abudance, these progenitors represent a potential cellular substrate for therapeutic transplantation. Nonetheless, only a few studies, constrained by the lack of any reliable method to isolate these cells, have attempted to assess the myelinogenic capacity of OPCs derived from the adult human white matter. In one such study (Targett *et al.*, 1996), a crude cell preparation derived from adult human white matter was transplanted into the ethidium bromide-lesioned and radiosensitized, X-irradiated adult rat spinal cord. The transplanted oligodendrocytes survived in the demyelinated zone, associated with denuded host axons, and expressed myelin proteins. But the transplanted cells did not migrate or divide, nor was any myelination noted. The failure of these implanted oligodendrocytes to myelinate was attributed to the diminished regenerative potential of post-mitotic oligodendrocytes, and the lack of a permissive environment for remyelination within the rat lesion bed (Targett *et al.*, 1996).

Propagated Oligospheres

Though neural stem cells have myelinogenic capacity, they also have the inherent capacity to generate neurons and astrocytes. The co-generation of astrocytes may not necessarily be deleterious, given their roles in both OPC proliferation and myelination (Blakemore, 1992; Franklin *et al.*, 1991). However, the co-generation of neurons may be undesirable, given the potential generation of ectopic neuronal foci, which might conceivably act as epileptogenic foci. Thus, priming neural stem cells or OPCs toward oligodendrocytic differentiation prior to implant might be necessary to ensure the quantities and phenotypic homogeneity of oligodendrocyte progenitor cells that will be needed for clinical implantation. One approach to this goal has been the expansion of neural stem cells as neurospheres in the presence of oligodendrocyte-inducing agents. For instance, when rat cerebellum-derived neurospheres were propagated in the presence of conditioned medium from the neuroblastoma B104 line (B104/CM), oligodendrocytes were preferentially generated. The resultant "oligospheres" were capable of being exponentially expanded through several passages without phenotypic degradation and exhibited robust myelination on transplantation into the *shiverer* mice brain (Avellana-Adalid *et al.*, 1996). Since then, several groups have used a similar strategy to generate oligospheres from neural precursor cells of the mouse, rat, and canine forebrains (Vitry *et al.*, 1999; Zhang *et al.*, 1998). Smith and Blakemore the compared the remyelinating capacity of cells isolated from porcine SVZ within hours after dissociation, to that exhibited by matched cells after growth in B104/CM as oligospheres. Whereas the freshly isolated SVZ cells remained undifferentiated after xenograft, those expanded in B104/CM effected significant remyelination of demyelinated axons *in vivo* (Smith and Blakemore, 2000).

Human OPCs Integrate When Grafted to Demyelinated Foci of the Adult Rat Brain

The remyelinating potential of adult human white matter-derived progenitors has been recently shown in lysolecithin-induced demyelinating lesions of adult rat corpus callosum

(Windrem *et al.*, 2002). In this study, A2B5 expression by P/CNP2:hGP-defined OPCs (Roy *et al.*, 1999) provided the rationale for immunomagnetically selecting OPCs on the basis of A2B5 expression. Like P/CNP2:hGFP$^+$ cells, A2B5-sorted cells generated largely oligodendrocytes when raised at high density in the presence of serum. In addition, immunomagnetic selection allowed their higher-yield acquisition, without the losses in viability and number associated with FACS separation. As a result, A2B5-antibody based immunomagnetic sorting increased the yield of extractable OPCs by over 5-fold. These A2B5-sorted white matter progenitors were transplanted into cyclosporine-immunosuppressed adult rats, 3 days after lysolecithin lesions. As previously described (Gensert and Goldman, 1997), these lesions yielded a discrete region of transcallosal demyelination, with mild local reactive astrocytosis within the demyelinated focus, and intact vasculature. When A2B5-sorted human OPCs were injected into these lesions, they migrated widely and rapidly; within 7 days of implantation, the cells had readily traversed the midline to infiltrate the furthest reaches of the demyelinated lesion beds, which often extended over 8 mm in breadth. The migration rate of the cells was hence at least 1 mm/day, or 50 μm per hour, within the lesion borders (Fig. 10.5). The engrafted adult A2B5-sorted progenitors differentiated rapidly, expressing CNP within 2 weeks and MBP within 3 weeks of implantation. These OPC-derived oligodendrocytes projected MBP$^+$ lamellopodia and were associated with a branched array of myelinating fibers, indicating the initiation of progenitor-associated myelinogenesis. Of note, many transplanted progenitor derived astrocytes were also observed in the lesions. With cyclosporine immunosuppression, the cells could survive at least 2 months in lysolecithin-demyelinated recipients. These findings suggested that the introduction of highly enriched preparations of progenitor cells derived from the adult human white matter might permit local remyelination.

Migratory Characteristics of human OPCs

Adult human-derived OPCs engrafted into demyelinated brain remained restricted to regions of demyelination; they rarely extended into normal surrounding myelin (Fig. 10.5). Even the few cells that were typically noted to have infiltrated normal myelin appeared to have migrated along the extraluminal surfaces of penetrating blood vessels. Yet when lentiviral-GFP tagged A2B5-sorted progenitor cell pools from adult human white matter were implanted into intact subcortex of adult rats, the transplanted cells remained localized to the implant site and continued to be so even after 3 months (Windrem *et al.*, 2002). These observations suggest strongly that normal adult white matter is non-permissive for the migration of adult-derived WMPCs, as has been observed in other studies (Iwashita *et al.*, 2000). This restriction on migration may be similar at the molecular level to that observed toward axons, whose extension through normal white matter is suppressed by their expression of Nogo receptor, by which they respond to myelin-associated Nogo and MAG (myelin-associated glycoprotein) with repulsion and/or cessation of further advance (Grandpre and Strittmatter, 2001). That being said, the operative white matter signals that restrict progenitor cell migration have yet to be identified. Whatever its mechanism, normal myelin clearly retains cues sufficient to tonically impede WMPC infiltration; accordingly, demyelination appears to remove those cues, allowing the active invasion and dispersion of OPCs throughout regions of acute myelin loss. The characterization of the ligands providing these repulsive cues, and of their anticipated progenitor cell receptors, will likely constitute an important avenue of future study.

Myelin Construction by Perinatal Transplant-Based Therapy

Several models of congenital dysmyelination have been used to assess the myelinogenic potential of animal and human-derived progenitors. The myelinogenic potential of implanted fetal human brain cells was first noted in the shiverer mouse (Gumpel *et al.*, 1987; Lachapelle *et al.*, 1983). The myelinogenic potential of different, stage-defined phenotypes of oligodendrocyte progenitors, extracted so as to sample the engraftment efficacy of different stages of progenitor progression, have also been compared in shiverer mice. Using rat donor tissue, Warrington and Pfeiffer established that the A2B5-defined oligo-

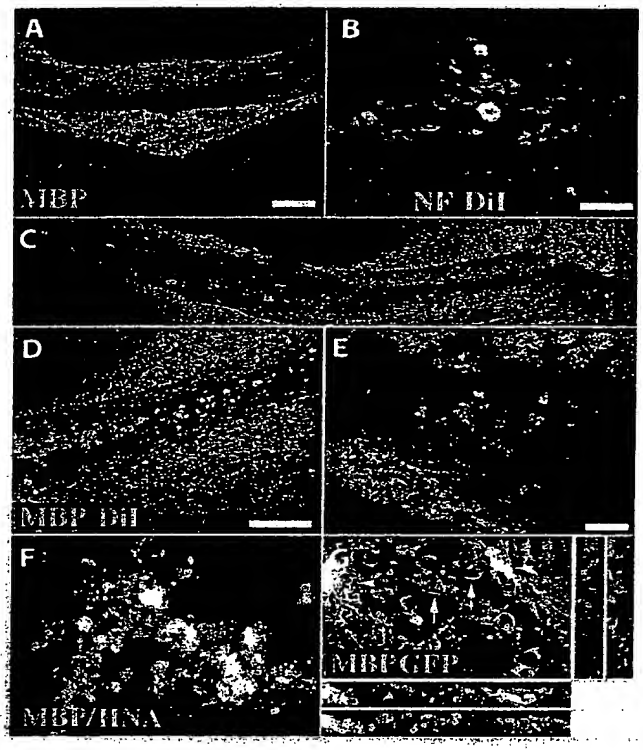

FIGURE 10.5
Implanted white matter progenitors migrated widely throughout the demyelinated callosum. Sorted adult human white matter progenitors were transplanted into lysolecithin-induced demyelinated lesions in the corpus callosa of adult rats. (A) Lysolecithin infusion (1 µl of 2% lysolecithin-V, delivered into the corpus callosum) yielded demyelinated plaques in the target white matter. In part A, the lesion is visible as a discoid region of myelin basic protein (MBP)-immunonegativity, surrounded by the otherwise MBP^+ callosum (green). (B) Though denuded of myelin (MBP, blue), $neurofilament^+$ axons (green) initially survived lysolecithin demyelination, 1 week after callosal lesion. The implanted progenitors (orange) have just immigrated to the lesion bed. Axonal spheroids were frequent within the lysolecithin-lesion bed, indicating some degree of early injury and transection. The ability of implanted progenitors to effect repair is limited by the viability and integrity of the axonal cohort that one wishes to myelinate. (C) This low-power montage demonstrates the rapidity of long-distance migration by xenografted adult human white matter progenitors. These DiI-labeled human progenitor cells (red) were visualized 1 week after their implantation, by which point the cells extend throughout the demyelinated lesion, defined by its loss of myelin basic protein (MBP)-immunoreactivity (green). The lesion was induced 3 days before 10^5 sorted, DiI-tagged (red) human progenitors were delivered in 2 µl. Within a week of implantation into this demyelinated callosum, the cells had traversed the midline. (D) A higher magnification image showing that the transplanted cells migrated throughout the demyelinated plaque, but not beyond its borders, except for occasional migrants that followed the parenchymal surfaces of blood vessels (arrow). The restriction of migration to demyelinated regions suggests that normal myelin impedes the migration of these cells. (E) Human white matter progenitor cells, identified as human nuclear $antigen^+$ (HNA; green), occupied the MBP (green)-deficient lysolecithin lesion, and expressed oligodendrocytic CNP (red) by 15 days after implantation. (F) A cluster of HNA^+ human cells (green) associated with a plethora of donor-derived MBP^+, myelinating oligodendrocytic lamellopodia (red). (G) Lentiviral GFP-tagged human (green) MBP^+ (red) oligodendrocytes in the lesion bed of a lysolecithin-injected rat callosum, 8 weeks after cell implantation. Besides the MBP^+ cells (arrows), other human progenitor-derived cells were also present, which did not express MBP and which instead manifested astrocytic morphologies (arrowheads). Immunolabeling adjacent sections for human GFAP (red) revealed that many of GFP-tagged human progenitors had also given rise to astrocytes. From Windrem *et al.* (2002). Scale bars: A, 200 µm; B, 20 µm; D, 100 µm; E, 30 µm.

dendrocyte progenitor phenotype was more efficient at migration and myelinogenesis in neonatal shiverers than the more mature O4-defined oligodendrocyte (Warrington *et al.*, 1993). Yandava *et al.* similarly achieved myelination within the shiverer brain, using the C17.2 line of transformed murine cerebellar progenitor cells, which act as neural stem cells after v-myc immortalization (Yandava *et al.*, 1999).

Similarly, fetal oligodendrocytes transplanted to the *md* rat remyelinated significant portions of the postnatal spinal cord (Archer *et al.*, 1994). Moreover, analogous studies in the *shaking* pup showed that fetal oligodendrocytes were able to engraft widespread regions of the *shaking* CNS, with graft survival of over 6 months. Although neonatal recipients fared best, adult recipients also exhibited graft oligodendrocyte survival and stable myelination (Archer *et al.*, 1997). Duncan and colleagues then demonstrated that oligosphere-derived cells raised from the neonatal rodent subventricular zone could engraft another dysmyelinated mutant, the myelin-deficient rat, upon perinatal intraventricular administration (Learish *et al.*, 1999). The success of these approaches led then to the seminal work of Mitome and colleagues, who used EGF responsive primary neural progenitor cells, in tandem with a combination of ventricular and cisternal transplant, to achieve the widespread myelination of the shiverer brain (Mitome *et al.*, 2001).

Human OPCs Can Myelinate Congenitally Dysmyelinated Brain

On the basis of these studies, Windrem *et al.* investigated whether highly enriched populations of human progenitor cells, directly isolated from the brain, might be used for cell-based therapy of congenital dysmyelination. Specifically, this study postulated that the efficiency of myelination might be improved by using purified OPCs, derived via selection so as to exclude astrocytes, microglia, and vascular derivatives from the implanted pool. It further postulated that such purified human OPCs, both adult-derived and taken from the fetal brain during its period of maximal oligoneogenesis, would be sufficiently migratory and myelinogenic to mediate the widespread myelination of a perinatal host. To this end, A2B5-based FACS was used in conjunction with PSA-NCAM-dependent immunodepletion of neuronal derivatives, to prepare highly enriched dissociates of human OPCs, of both fetal and adult derivation. Both classes of human oligodendrocyte progenitor cells proved capable of widespread and high-efficiency myelination of the shiverer brain after perinatal xenograft. Indeed, the cells migrated so widely as to effect myelination throughout the recipient brains (Fig. 10.6, unpublished data). The cells infiltrated widely throughout the presumptive white matter, ensheathed resident murine axons, and formed antigenically and ultrastructurally compact myelin. After implantation, the cells slowed their mitotic expansion with time and generated neither undesired phenotypes nor parenchymal aggregates. In this initial study, despite histologically extensive myelination in these animals, no change in the behavioral phenotype of the shi/shi recipients or any improvement in their neurological phenotype was evident. Nonetheless, the geographic extent of forebrain and diencephalic MBP expression evidenced by these animals, who received but a single perinatal intraventricular cell injection, suggested that combined cisternal and intraventricular delivery of donor progenitors might achieve remyelination throughout the rostral neuraxis, potentially spanning the entire brain.

Besides demonstrating the myelinogenic capacity of the transplanted cells, studies in the dysmyelinated animal models indirectly indicate that congenital dysmyelination, even more so than adult demyelination, may be an appropriate target for CNS progenitor cell-based therapy. In particular, these studies affirmed that the neonatal brain environment may be especially amenable to therapeutic remyelination. It is conducive to widespread migration and may continue to provide the instructive developmental cues necessary for region-specific differentiation.

Fetal and Adult OPCs Differ

Despite the use of both fetal and adult-derived OPCs in experimental therapeutic models, no head-to-head comparison of the two phenotypes had ever been performed from

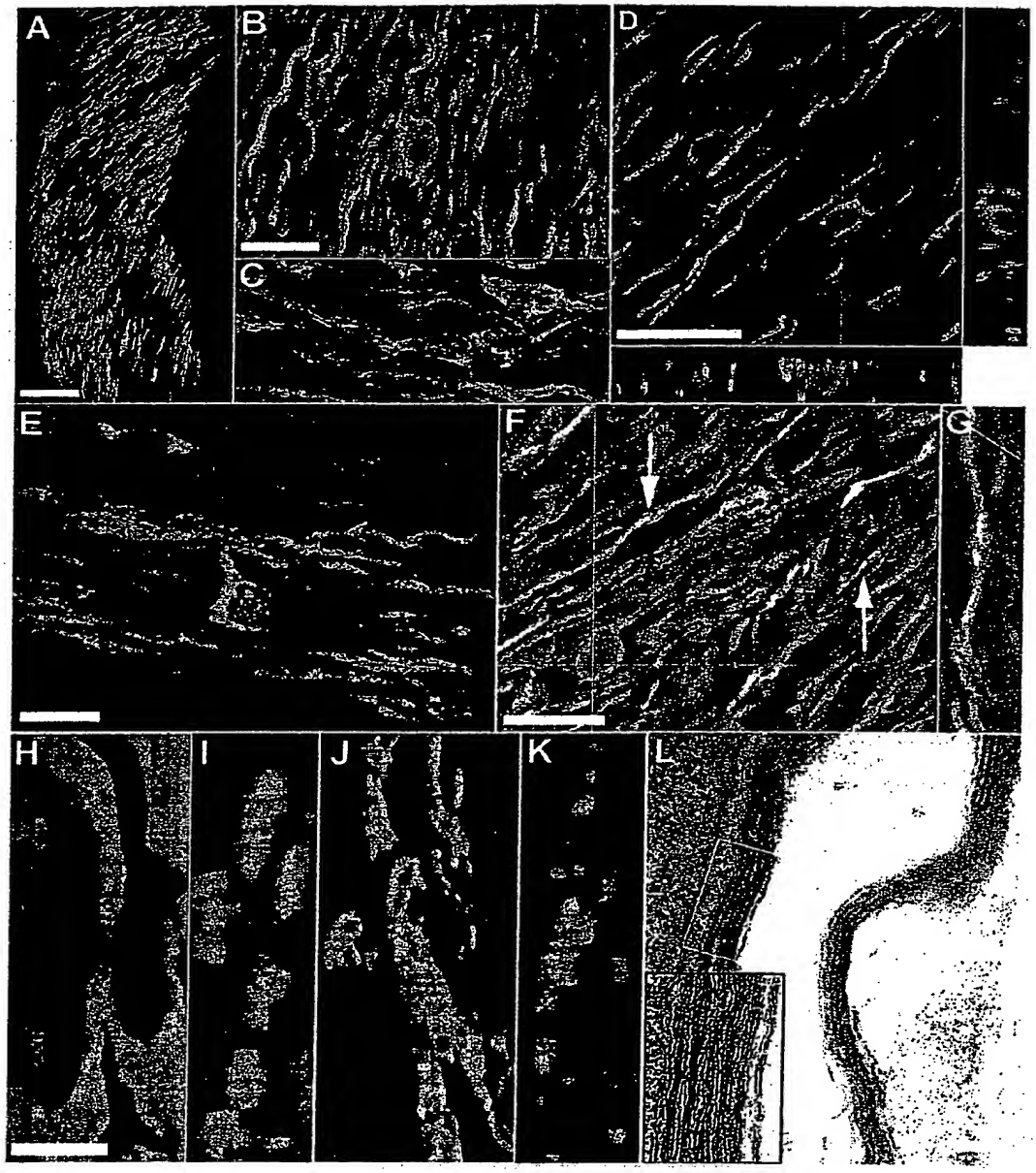

FIGURE 10.6

Myelin basic protein (MBP) was widely expressed by human fetal OPCs implanted into neonatal homozygote shiverer mice. (A) This low-power view of the recipient fimbria shows abundant fiber-associated MBP expression (*green*), 3 months after perinatal engraftment (MBP, *green*) Since shiverer homozygotes do not express immunoreactive MBP, all such signal must derive from donor progenitor cells. (B-D) Donor fetal OPCs, additionally validated as such by human nuclear antigen (hNA, *red*), differentiated to express CNP protein (*green* in B) and MBP (*green* in C-D). In the 0.5 μm confocal optical section of D, MBP (*green*) is noted to surround the donor human nucleus (*red*), as viewed in orthogonal planes. (E). A single donor-derived MBP^+ oligodendrocyte that has matured, 3 months after engraftment, to associate with multiple recipient axons. (F) An 0.2 μm optical section through a recipient corpus callosum shows engrafted human OPCs (hNA, *blue*) expressing MBP (*red*), and surrounding native axons (neurofilament, *green*). Arrows indicate examples of ensheathed axons, a higher magnification of which is shown in (G). Human OPCs enwrap native axons and reorganize the paranodal region to permit nodal formation. Caspr protein, an axonal paranodal marker, is expressed on unmyelinated axons between myelinated segments of axon, without invading the nodal region. (H-K) Optical sections through engrafted shiverer corpus callosum, showing donor-derived MBP (*green*) and native axonal Caspr protein in red, indicating that donor OPCs develop not only myelin production and architecture, but permit nodes of Ranvier to form (anti-Caspr antibodies generously provided by Dr. M. Rasband). (L) Electron microscopy confirmed that donor-derived oligodendrocytes developed compact myelin, I that myelin produced by engrafted fetal human OPCs wrapped native axons to form compact sheathes with major dense lines (*inset*). Scale bars: A, 200μm; B-F, 20μm; H-K, 5 μm; L, 1 μm.

analogously acquired and maintained cells implanted into the same models at the same time. As a result, it was unclear if fetal-derived OPCs differed from their counterparts derived from the adult human brain, with respect to either their migration competence, myelinogenic capacity, or the time courses thereof. To assess the relative advantages and disadvantages as therapeutic vectors of these two stage-defined OPC phenotypes, newborn shiverer mice were implanted with either fetal or adult-derived OPCs, each isolated via A2B5-directed immunomagnetic sorting (IMS). The implanted neonatal mice were allowed to survive for 1 to 3 months, and their brains then sectioned and stained for MBP, GFAP, and anti-human nuclear antigen. By this means, it was determined that fetal and adult-derived human OPCs differed substantially in their respective time courses and efficacy of myelinogenesis upon xenograft. Adult OPCs myelinated shiverer brain more rapidly than their fetal counterparts, achieving widespread and dense MBP expression by 4 weeks after xenograft. In contrast, substantial MBP expression by fetal OPCs was generally not observed until 12 weeks post-implant (Windrem *et al.*, unpublished data).

Besides myelinating more quickly than fetal OPCs, adult OPCs were found to give rise to myelinogenic oligodendrocytes in much higher relative proportions, and with much less astrocytic co-generation, than did fetal-derived OPCs. When assessed at the midline of the recipient corpus callosum, just over 10% of fetal hNA-defined OPCs expressed MBP at 12 weeks, while virtually none had done so at 4 weeks. In contrast, almost 40% of adult OPCs expressed MBP by 4 weeks after xenograft into matched recipients. Thus, engrafted adult OPCs were at least four times more likely to mature as oligodendrocytes and develop myelin than their fetal counterparts. As another cardinal difference between fetal and adult OPCs, adult OPCs largely remained restricted to the host white matter, within which they generated almost entirely MBP^+ oligodendrocytes. In contrast, fetal OPCs migrated into both gray and white matter, generating both astrocytes and oligodendrocytes in a context-dependent manner.

Thus, both fetal and adult-derived OPCs were competent to remyelinate murine axons, but each had distinct advantages and disadvantages as potential vectors for cell therapy: Whereas fetal OPCs were highly migratory, they myelinated slowly and inefficiently. In contrast, adult-derived OPCs migrated over lesser distances, but they myelinated more rapidly and in higher proportions than their fetal counterparts. Together, these studies argued that while both fetal and adult human OPCs might provide effective cellular substrates for remyelination, the choice of cellular source must be dictated not only by the availability of donor material, but also by the specific biology of the disease target.

A Caveat: Some Implanted Progenitors May Remain Undifferentiated

A corollary of the multipotential nature of white matter progenitor cells is that when transplanted as nominally oligodendrocytic precursors, these cells might encounter local signals that instruct their maturation along alternative lineages. As a result, we need to be concerned about the possibility of their differentiation into undesired or functionally heterotopic phenotypes. This possibility is of further concern given the persistence of many implanted progenitors as undifferentiated cells; these may remain able to respond to signals in the host tissue environment, not only at the time of implantation, but also long thereafter. As such, these cells might comprise a reservoir of implanted precursors, from which desired phenotypes might be later recruited upon injury or insult. On the other hand, they might just as well constitute potential sources of undesired cell types that might be ectopically generated and recruited in the tissue environment of an acutely injured focus. Such local production of undesired phenotypes might introduce not only inefficiency to transplant-based treatment strategies, but also frank danger. For instance, the production of neurons in a white matter lesion could generate an epileptogenic focus, just as the production of astrocytes in a more typically oligodendrocytic region might disrupt local ionic gradients and hence axonal transmission. These and many other untoward processes of heterotopic phenotypic maturation could more than offset whatever benefits might be gleaned from a therapeutic cell implant. As a result, it may prove advisable to initiate the phenotypic differentiation of these cells *in vitro*, prior to implantation, so as to limit

the range of phenotypic choices available to the isolated progenitors to those appropriate for the intended region and disease target. Time will tell whether the possibility of heterotopic misdifferentiation will mandate such *in vitro* priming steps.

EXPERIMENTAL IMPLANTATION OF NON-CNS PROGENITOR CELL TYPES

A wide range of other potentially myelinogenic cell types have also been implanted into experimental models of de- and dysmyelination, with varying degrees of success.

Schwann Cells

Schwann cells, the myelinating cells of the peripheral nervous system, have been considered as an attractive alternative to oligodendrocyte precursors for experimental transplantation. Schwann cells from several sources, including humans (Kohama *et al.*, 2001), have been implanted in dysmyelinated *shiverer* mice (Baron-Van Evercooren *et al.*, 1992), MD rats, and *shaking* pups (Duncan and Hoffman, 1997). They have also been transplanted into lysolecithin (Baron-Van Evercooren *et al.*, 1993; Duncan *et al.*, 1981) and EB-X (Blakemore and Crang, 1985) demyelinated lesions in the brain and spinal cord. In all these systems, they have demonstrated varying degrees of myelination (Franklin and Barnett, 1997) with the myelin produced by these cells being of the PNS-variety as specified by the expression of P0. In some cases, functional reconstitution of saltatory conduction has also been shown (Felts and Smith, 1992; Honmou *et al.*, 1996; Kohama *et al.*, 2001). In addition, Schwann cells have been reported to improve axonal regeneration, which might be of importance in MS where axonal loss is a major part of the lesion pathology. Considering the relative ease of expanding human Schwann cells in culture (Rutkowski *et al.*, 1995), it has been suggested that they might be appropriate cellular vectors for autologous transplants. Indeed, they have the added advantage of producing non-CNS myelin, which may be refractory to the immunological destruction in diseases like MS. However, like central oligodendrocyte progenitors, the migratory capacity of these cells is unclear. Some studies indicate that Schwann cells migrate satisfactorily over large distances to specific target sites (Franklin and Barnett, 1997), while others indicate that they are unable to migrate through normal white matter (Iwashita *et al.*, 2000). In addition, Schwann cells seem to have a complex relationship with central astrocytes. After transplantation, Schwann cells are mainly found in areas devoid of astrocytes (Baron-van Evercooren *et al.*, 1992; Blakemore and Patterson, 1975), and, moreover, they are excluded as astrocyte numbers increase with time (Shields *et al.*, 2000).

Olfactory Ensheathing Cells (OEC)

In nature, OEC's ensheath small diameter axons of the peripheral olfactory epithelium neurons that project through the olfactory nerve into the olfactory bulb of the CNS. Unlike Schwann cells, these cells do not normally produce myelin. However, OECs from both animal (Franklin *et al.*, 1996; Imaizumi *et al.*, 1998) and human sources (Barnett *et al.*, 2000; Kato *et al.*, 2000) show remyelination with a peripheral pattern of myelin expression upon transplantation to demyelinated spinal cords. In some studies, a functional restoration of conduction has also been demonstrated (Imaizumi *et al.*, 2000). OECs may have an advantage over Schwann cells, as they co-exist naturally with astrocytes in the olfactory bulb (Lakatos *et al.*, 2000). Perhaps as a result, their interaction with astrocytes in not restrictive (Franklin and Barnett, 2000) in fact, they have been reported to support axonal regeneration through the astrocytic environment of a transected spinal cord (Ramon-Cueto *et al.*, 1998). Nonetheless, their restoration of central axonal conduction remains inconclusive, as is the long-term fate of their remyelinated units. Whether these cells are capable of the contact-dependent and humoral support of neuronal function

normally exercised by central oligodendrocytes, or conversely, whether they are in turn supported by the axons with which they interact (Fernandez *et al.*, 2000; Vartanian *et al.*, 1997), similarly remain unknown.

Embryonic Stem Cells

Myelination by *in vitro* conditioned mouse embryonic stem cells has been reported in both hypomyelinated MD rat E-17 fetuses and *shiverer* newborns, as well as in adult lysolecithin demyelinated lesions in adult rats (Brustle *et al.*, 1999; Liu *et al.*, 2000). More recent reports describe transplanted human ES cells sequentially cultured to induce neural stem cells capable of generating oligodendrocytes in a region-specific manner (Reubinoff *et al.*, 2001). Though ES cells might represent a readily cultivable source of OPCs, the use of these cells is still limited by our inability to fully instruct all cells in the undifferentiated population to the desired phenotype. Of greater concern is the persistent uncommitted progenitors within the implanted population, which may retain the latent capacity for undifferentiated expansion and possibly tumorigenicity.

Mesenchymal and Marrow-Derived Stem Cells

In addition to ES cells, mesenchymal and marrow-derived stem cells have been in focus as a source of neurally specified cells. Some controversial studies indicate that these cells may be capable of trans- or ectopic differentiation to neuroectodermal lineage (Mezey *et al.*, 2000; Sanchez-Ramos *et al.*, 2000). Of particular concern has been the lack of clear clonal evidence of neural specification as well as recent reports indicting that cell fusion may explain some of observations of trans-differentiation (Terada *et al.*, 2002; Ying *et al.*, 2002). Nonetheless, a recent study, in which mouse bone marrow stromal cells were grafted into EB-X demyelinated spinal cord lesions, reported not only donor-cell derived histological remyelination, but also an improvement in conduction velocity (Akiyama *et al.*, 2002). This work remains to be replicated by other groups. Should this study prove verifiable, its approach may open new avenues of stromal cell-based remyelination therapy.

DISEASE TARGETS FOR PROGENITOR-BASED THERAPEUTIC MYELINATION

Congenital Dysmyelination

Congenital diseases of myelination, such as periventricular leukomalacia (PVL), which may serve as an anatomic form fruste for the later development of cerebral palsy (Grow and Barks, 2002; Rezaie and Dean, 2002; Volpe, 2001) and the hereditary leukodystrophies and storage diseases, such as Krabbe's and Tay Sachs disease, are leading causes of infant morbidity and mortality (reviewed by Schiffmann and Boespflug-Tanguy, 2001; Berger *et al.*, 2001). As such, these may constitute feasible and attractive targets for therapeutic remyelination (Tate *et al.*, 2001).

Periventricular leukomalacia PVL describes a lesion of the periventricular white matter, associated with a failure in early myelination of the cerebral hemispheres. PVL appears to be a pathological concomitant to perinatal hypoxic-ischemic insult and may result from germinal matrix hemorrhage, sustained hypoxia, and excitotoxic injury, and most likely from combinations of these insults. PVL predicts the development of cerebral palsy in most cases (Volpe, 2001). Experimental models of hypoxic-ischemia in neonatal rats (Back *et al.*, 2002; Levison *et al.*, 2001) as well as studies of pediatric autopsies (Back *et al.*, 2001) have suggested that the late oligodendrocyte progenitors of the forebrain SVZ comprise the predominant cell population lost in perinatal ischemic injury. This is in accord with our understanding of the natural history of oligodendrogliogenesis in humans (Grever *et al.*, 1999; Rakic and Zecevic, 2003; Zhang *et al.*, 2000), the developmental window for which corresponds to the period of ischemic vulnerability of the periventricular white matter.

Congenital leukodystrophies include an ever-expanding group of inherited diseases of myelin synthesis and metabolism. Although a diverse group, these may roughly be divided into lysosomal storage diseases, such as Krabbe's globoid cell leukodystrophy (Wenger *et al.*, 2000) and Tay Sachs diseases (Gravel *et al.*, 1991); disorders of myelin synthesis, such as Pelizaeus-Merbacher disease (PMD) (Koeppen and Robitaille, 2002); and metabolic deficiencies leading to toxic demyelination, such as Canavan's disease (Matalon and Michals-Matalon, 2000). Each of these disease categories is attended by extensive white matter involvement and clinical leukoencephalopathy, typically leading to severe neurological disability and death. As a group, the clinical leukodystrophies represent especially attractive targets for progenitor cell-based therapy, since the restoration of healthy oligodendrocytes in early perinatal development may be sufficient to permit myelination and hence to slow or prevent the development of the disease phenotype. In addition, effective murine models of these diseases are available (Werner *et al.*, 1998). Inherited diseases of the PLP and MBP genes are modeled by twitcher (Mikoshiba *et al.*, 1985; Yoshimura *et al.*, 1989) and shiverer mice (Roach *et al.*, 1985), respectively. In addition, mutations of hexoseaminidase-B, modeling Sandhoff's and Tay-Sachs diseases (Kolter and Sandhoff, 1998), and aspartoacylase, mimicking Canavan's disease (Matalon *et al.*, 2000), have been similarly employed. The availability of such genetically precise models of the childhood leukodystrophies is already greatly accelerating the process of developing experimental treatment strategies for these disorders.

Acquired Demyelination

In adults, the diseases of acquired demyelination include later-onset leukodystrophies, such as metachromatic leukodystrophy and adrenoleukodystrophy, as well as vascular, inflammatory, and nutritional demyelinating syndromes (Baumann and Turpin, 2000; Berger *et al.*, 2001; Desmond, 2002; Dichgans, 2002). The vascular demyelinations include hypertensive and diabetic leukoencephalopathies, which may both be due to chronic oligodendrocytic ischemic hypoxia (Dewar *et al.*, 1999; Leys *et al.*, 1999). Subcortical strokes, particularly those within the distributions of the forebrain lenticulostriate and thalamogeniculate arterial territories, are also prominent causes of vascular demyelination (Dichgans, 2002). The inflammatory demyelinations include multiple sclerosis, transverse myelitis (Kerr and Ayetey, 2002), optic neuritis (Cree *et al.*, 2002; Eggenberger, 2001), and less commonly Schilder's leukoencephalitis (Kotil *et al.*, 2002), as well as postvaccinial (An *et al.*, 2002; Konstantinou *et al.*, 2001) and postinfectious leukoencephalitis (Kleinschmidt-DeMasters and Gilden, 2001; Rust, 2000). All of these syndromes of acquired demyelination are potential targets of therapeutic remyelination. Yet most attempts at cell-based remyelination in experimental animals have been made using acute chemical demyelinating insults, such as lysolecithin. In contrast to the availability of effective animal models of congenital dysmyelination, the study of acquired demyelination has suffered from its lack of biologically appropriate, clinically reflective animal models. As a result, few adequate studies of cell-based remyelination of acquired, adult demyelinating lesions have been reported using any cellular vector. Those studies that have reported oligodendrocytic maturation and myelination by implanted oligodendrocyte progenitors have typically failed to demonstrate substantial axonal ensheathment, though this has likely reflected the loss of competent axons in these models, rather than any insufficiency on the part of the implanted progenitor cells. Indeed, the etiological complexity and manifold sequelae of demyelination in the adult brain argues against easy therapeutic intervention. As such, until improved models of acquired demyelinating disease are available, progress in cell-based therapy of adult demyelinating diseases will be necessarily slow. In contrast, the arguably simpler etiologies of congenital dysmyelination, their frequent lack of association with underlying systemic disease, and the persistent structural plasticity of the perinatal brain, together with the many effective animal models for congenital dysmyelination, collaborate to make these diseases attractive targets for near-term intervention, both experimentally and clinically. Indeed, we may reasonably except the congenital leukodystrophies to be especially promising targets for cell-based therapeutic remyelination.

References

Ader, M., Schachner, M., and Bartsch, U. (2001). Transplantation of neural precursor cells into the dysmyelinated CNS of mutant mice deficient in the myelin-associated glycoprotein and Fyn tyrosine kinase. Eur J Neurosci **14**, 561–566.

Adlkofer, K., and C., L. (2000). Role of neuregulins in glial cell development. Glia **29**, 104–111.

Akiyama, Y., Honmou, O., Kato, T., Uede, T., Hashi, K., and Kocsis, J. D. (2001). Transplantation of clonal neural precursor cells derived from adult human brain establishes functional peripheral myelin in the rat spinal cord. Exp Neurol **167**, 27–39.

Akiyama, Y., Radtke, C., and Kocsis, J. D. (2002). Remyelination of the rat spinal cord by transplantation of identified bone marrow stromal cells. J Neurosci **22**, 6623–6630.

Aloisi, F., Giampaolo, A., Russo, G., Peschle, C., and Levi, G. (1992). Developmental appearance, antigenic profile, and proliferation of glial cells of the human embryonic spinal cord: An immunocytochemical study using dissociated cultured cells. Glia **5**, 171–181.

Altman, J., and Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J. Comparative Neurology **124**, 319–335.

Altman, J., and Das, G. D. (1966). Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. J. Comp. Neurol. **127**, 337–390.

An, S. F., Groves, M., Martinian, L., Kuo, L. T., and Scaravilli, F. (2002). Detection of infectious agents in brain of patients with acute hemorrhagic leukoencephalitis. J Neurovirol. **8**, 439–446.

Archer, D., Cuddon, P., Lipsitz, D., and Duncan, I. (1997). Myelination of the canine central nervous sytem by glial cell transplantation: A model for repair of human myelin disease. Nature Medicine **3**, 54–59.

Archer, D. R., Leven, S., and Duncan, I. D. (1994). Myelination by cryopreserved xenografts and allografts in the myelin-deficient rat. Exp Neurol **125**, 268–277.

Armstrong, R. C., Dorn, H. H., Kufta, C. V., Friedman, E., and Dubois-Dalcq, M. E. (1992). Pre-oligodendrocytes from adult human CNS. J Neurosci **12**, 1538–1547.

Avellana-Adalid, V., Nait-Oumesmar, B., Lachapelle, F., and Baron-Van Evercooren, A. (1996). Expansion of rat oligodendrocyte progenitors into proliferative "oligospheres" that retain differentiation potential. J Neurosci Res **45**, 558–570.

Baas, D., Bourbeau, D., Sarlieve, L. L., Ittel, M. E., Dussault, J. H., and Puymirat, J. (1997). Oligodendrocyte maturation and progenitor cell proliferation are independently regulated by thyroid hormone. Glia **19**, 324–332.

Bach, M. A., Shen-Orr, Z., Lowe, W. L., Jr., Roberts, C. T., Jr., and LeRoith, D. (1991). Insulin-like growth factor I mRNA levels are developmentally regulated in specific regions of the rat brain. Brain Res Mol Brain Res **10**, 43–8.

Back, S. A., Han, B. H., Luo, N. L., Chricton, C. A., Xanthoudakis, S., Tam, J., Arvin, K. L., and Holtzman, D. M. (2002). Selective vulnerability of late oligodendrocyte progenitors to hypoxia-ischemia. J Neuroscience **22**, 455–463.

Back, S. A., Luo, N. L., Borenstein, N. S., Levine, J. M., Volpe, J. J., and Kinney, H. C. (2001). Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. J Neurosci **21**, 1302–1312.

Bansal, R., Kumar, M., Murray, K., Morrison, R. S., and Pfeiffer, S. E. (1996). Regulation of FGF receptors in the oligodendrocyte lineage. Mol Cell Neurosci **7**, 263–275.

Bansal, R., and Pfeiffer, S. E. (1994). Regulation of gene expression in mature oligodendrocytes by the specialized myelin-like membrane environment: Antibody perturbation in culture with the monoclonal antibody R-mAb. Glia **12**, 173–179.

Bansal, R., and Pfeiffer, S. E. (1997). FGF-2 converts mature oligodendrocytes to a novel phenotype. J Neurosci Res **50**, 215–228.

Barami, K., Iversen, K., Furneaux, H., and Goldman, S. A. (1995). Hu protein as an early marker of neuronal phenotypic differentiation by subependymal zone cells of the adult songbird forebrain. J Neurobiol **28**, 82–101.

Barami, K., Zhao, J., Diaz, F. G., and Lyman, W. D. (2001). Comparison of neural precursor cell fate in second trimester human brain and spinal cord. Neurol Res **23**, 260–266.

Barnett, S. C., Alexander, C. L., Iwashita, Y., Gilson, J. M., Crowther, J., Clark, L., Dunn, L. T., Papanastassiou, V., Kennedy, P. G., and Franklin, R. J. (2000). Identification of a human olfactory ensheathing cell that can effect transplant-mediated remyelination of demyelinated CNS axons. Brain **123**, 1581–1588.

Baron-Van Evercooren, A., Duhamel-Clerin, E., Boutry, J. M., Hauw, J. J., and Gumpel, M. (1993). Pathways of migration of transplanted Schwann cells in the demyelinated mouse spinal cord. J Neurosci Res **35**, 428–438.

Baron-Van Evercooren, A., Gansmuller, A., Duhamel, E., Pascal, F., and Gumpel, M. (1992). Repair of a myelin lesion by Schwann cells transplanted in the adult mouse spinal cord. J Neuroimmunol **40**, 235–242.

Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyvodic, J. T., Richardson, W. D., and Raff, M. C. (1992). Cell death and control of cell survival in the oligodendrocyte lineage. Cell **70**, 31–46.

Barres, B. A., Lazar, M. A., and Raff, M. C. (1994a). A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development **120**, 1097–1108.

Barres, B. A., Raff, M. C., Gaese, F., Bartke, I., Dechant, G., and Barde, Y. A. (1994b). A crucial role for neurotrophin-3 in oligodendrocyte development. Nature **367**, 371–375.

Baumann, N., and Turpin, J. C. (2000). Adult-onset leukodystrophies. J Neurology 247, 751–759.
Berger, J., Moser, H. W., and Forss-Petter, S. (2001). Leukodystrophies: Recent developments in genetics, molecular biology, pathogenesis and treatment. Current Opinion in Neurology 14, 305–312.
Besnard, F., Perraud, F., Sensenbrenner, M., and Labourdette, G. (1989). Effects of acidic and basic fibroblast growth factors on proliferation and maturation of cultured rat oligodendrocytes. Int J Dev Neurosci 7, 401–409.
Blakemore, W. F. (1992). Transplanted cultured type-1 astrocytes can be used to reconstitute the glia limitans of the CNS: The structure which prevents Schwann cells from myelinating CNS axons. Neuropathol Appl Neurobiol 18, 460–466.
Blakemore, W. F., and Crang, A. J. (1985). The use of cultured autologous Schwann cells to remyelinate areas of persistent demyelination in the central nervous system. J Neurol Sci 70, 207–223.
Blakemore, W. F., and Patterson, R. C. (1975). Observations on the interactions of Schwann cells and astrocytes following X-irradiation of neonatal rat spinal cord. J Neurocytol 4, 573–85.
Brustle, O., Choudhary, K., Karram, K., Huttner, A., Murray, K., Dubois-Dalcq, M., and McKay, R. D. (1998). Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats. Nat Biotechnol 16, 1040–4.
Brustle, O., Jones, K. N., Learish, R. D., Karram, K., Choudhary, K., Wiestler, O. D., Duncan, I. D., and McKay, R. D. (1999). Embryonic stem cell-derived glial precursors: A source of myelinating transplants. Science 285, 754–756.
Buchet, D., Buc-Caron, M. H., Sabate, O., Lachapelle, F., and Mallet, J. (2002). Long-term fate of human telencephalic progenitor cells grafted into the adult mouse brain: Effects of previous amplification in vitro. J Neurosci Res 68, 276–283.
Buonanno, A., and Fischbach, G. D. (2001). Neuregulin and ErbB receptor signaling pathways in the nervous system. Curr Opin Neurobiol. 11, 287–296.
Cannella, B., Pitt, D., Marchionni, M., and Raine, C. S. (1999). Neuregulin and erbB receptor expression in normal and diseased human white matter. J Neuroimmunol 100, 233–242.
Canoll, P. D., Kraemer, R., Teng, K. K., Marchionni, M. A., and Salzer, J. L. (1999). GGF/neuregulin induces a phenotypic reversion of oligodendrocytes. Mol Cell Neurosci 13, 79–94.
Canoll, P. D., Musacchio, J. M., Hardy, R., Reynolds, R., Marchionni, M. A., and Salzer, J. L. (1996). GGF/neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors. Neuron 17, 229–243.
Carpenter, M. K., Cui, X., Hu, Z. Y., Jackson, J., Sherman, S., Seiger, A., and Wahlberg, L. U. (1999). In vitro expansion of a multipotent population of human neural progenitor cells. Exp Neurol 158, 265–278.
Carroll, W. M., Jennings, A. R., and Ironside, L. J. (1998). Identification of the adult resting progenitor cell by autoradiographic tracking of oligodendrocyte precursors in experimental CNS demyelination. Brain 121, 293–302.
Carson, M., Behringer, R., Brinster, R., and McMorris, F. (1993). Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. Neuron 10, 729–740.
Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. Science 263, 802–805.
Chang, A., Tourtellotte, W. W., Rudick, R., and Trapp, B. D. (2002). Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. N Engl J Med 346, 165–173.
Chari, D. M., and Blakemore, W. F. (2002). Efficient recolonisation of progenitor-depleted areas of the CNS by adult oligodendrocyte progenitor cells. Glia 37, 307–13.
Cree, B. A., Goodin, D. S., and Hauser, S. L. (2002). Neuromyelitis optica. Seminars in Neurology 22, 105–22.
Davis, A. A., and Temple, S. (1994). A self-renewing multipotential stem cell in embryonic rat cerebral cortex. Nature 372, 263–6.
Dawson, M. R., Levine, J. M., and Reynolds, R. (2000). NG2-expressing cells in the central nervous system: Are they oligodendroglial progenitors? J Neurosci Res 61, 471–479.
Deadwyler, G. D., Pouly, S., Antel, J. P., and Devries, G. H. (2000). Neuregulins and erbB receptor expression in adult human oligodendrocytes. Glia 32, 304–312.
Desmond, D. W. (2002). Cognition and white matter lesions. Cerebrovascular Disease 13, 53–57.
Dewar, D., Yam, P., and McCulloch, J. (1999). Drug development for stroke: Importance of protecting cerebral white matter. European Journal of Pharmacology 375, 41–50.
Dichgans, M. (2002). Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy: Phenotypic and mutational spectrum. J Neurological Sciences *203–204*, 77–80.
Duncan, I. D., Aguayo, A. J., Bunge, R. P., and Wood, P. M. (1981). Transplantation of rat Schwann cells grown in tissue culture into the mouse spinal cord. J Neurol Sci 49, 241–252.
Duncan, I. D., and Hoffman, R. L. (1997). Schwann cell invasion of the central nervous system of the myelin mutants. J Anat 190, 35–49.
Eggenberger, E. R. (2001). Inflammatory optic neuropathies. Ophthalmol Clin North Am. 14, 73–82.
Eisenbarth, G. S., Walsh, F. S., and Nirenberg, M. (1979b). Monoclonal antibody to a plasma membrane antigen of neurons. Proc Natl Acad Sci USA 76, 4913–4917.
Ellison, J. A., and de Vellis, J. (1994). Platelet-derived growth factor receptor is expressed by cells in the early oligodendrocyte lineage. J Neurosci Res. 37, 116–128.
Engel, U., and Wolswijk, G. (1996). Oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells derived from adult rat spinal cord: In vitro characteristics and response to PDGF, bFGF and NT-3. Glia 16, 16–26.

Englund, U., Bjorklund, A., and Wictorin, K. (2002). Migration patterns and phenotypic differentiation of long-term expanded human neural progenitor cells after transplantation into the adult rat brain. Brain Res Dev Brain Res **134**, 123–141.

Eriksson, P., Perfilieva, E., Bjork-Eriksson, Alborn, A., Nordberg, C., Peterson, D., and Gage, F. (1998). Neurogenesis in the adult human hippocampus. Nature Medicine **4**, 1313–1317.

Felts, P. A., and Smith, K. J. (1992). Conduction properties of central nerve fibers remyelinated by Schwann cells. Brain Res **574**, 178–192.

Fernandez, P. A., Tang, D. G., Cheng, L., Prochiantz, A., Mudge, A. W., and Raff, M. C. (2000). Evidence that axon-derived neuregulin promotes oligodendrocyte survival in the developing rat optic nerve. Neuron **28**, 81–90.

Ffrench-Constant, C., and Raff, M. C. (1986). Proliferating bipotential glial progenitor cells in adult rat optic nerve. Nature **319**, 499–502.

Franklin, R. J., and Barnett, S. C. (1997). Do olfactory glia have advantages over Schwann cells for CNS repair? J Neurosci Res **50**, 665–672.

Franklin, R. J., and Barnett, S. C. (2000). Olfactory ensheathing cells and CNS regeneration: The sweet smell of success? Neuron **28**, 15–18.

Franklin, R. J., and Blakemore, W. F. (1997). To what extent is oligodendrocyte progenitor migration a limiting factor in the remyelination of multiple sclerosis lesions? Mult Scler **3**, 84–87.

Franklin, R. J., Crang, A. J., and Blakemore, W. F. (1991). Transplanted type-1 astrocytes facilitate repair of demyelinating lesions by host oligodendrocytes in adult rat spinal cord. J Neurocytol **20**, 420–430.

Franklin, R. J., Gilson, J. M., Franceschini, I. A., and Barnett, S. C. (1996). Schwann cell-like myelination following transplantation of an olfactory bulb-ensheathing cell line into areas of demyelination in the adult CNS. Glia **17**, 217–224.

Fricker, R. A., Carpenter, M. K., Winkler, C., Greco, C., Gates, M. A., and Bjorklund, A. (1999). Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. J Neurosci **19**, 5990–6005.

Fruttiger, M., Karlsson, L., Hall, A. C., Abramsson, A., Calver, A. R., Bostrom, H., Willetts, K., Bertold, C. H., Heath, J. K., Betsholtz, C., and Richardson, W. D. (1999). Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. Development **126**, 457–467.

Gage, F. H., Kempermann, G., Palmer, T. D., Peterson, D. A., and Ray, J. (1998). Multipotent progenitor cells in the adult dentate gyrus. J Neurobiol **36**, 249–266.

Gansmuller, A., Lachapelle, F., Baron-Van Evercooren, A., Hauw, J. J., Baumann, N., and Gumpel, M. (1986). Transplantations of newborn CNS fragments into the brain of shiverer mutant mice: Extensive myelination by transplanted oligodendrocytes. II. Electron microscopic study. Dev Neurosci **8**, 197–207.

Gensert, J. M., and Goldman, J. E. (1996). In vivo characterization of endogenous proliferating cells in adult rat subcortical white matter. Glia **17**, 39–51.

Gensert, J. M., and Goldman, J. E. (1997). Endogenous progenitors remyelinate demyelinated axons in the adult CNS. Neuron **19**, 197–203.

Gensert, J. M., and Goldman, J. E. (2001). Heterogeneity of cycling glial progenitors in the adult mammalian cortex and white matter. J Neurobiol **48**, 75–86.

Gloster, A., Wu, W., Speelman, A., Weiss, S., Causing, C., Pozniak, C., Reynolds, B., Chang, E., Toma, J. G., and Miller, F. D. (1994). The T alpha 1 alpha-tubulin promoter specifies gene expression as a function of neuronal growth and regeneration in transgenic mice. J Neurosci **14**, 7319–7330.

Gogate, N., Verma, L., Zhou, J. M., Milward, E., Rusten, R., O'Connor, M., Kufta, C., Kim, J., Hudson, L., and Dubois-Dalcq, M. (1994). Plasticity in the adult human oligodendrocyte lineage. J Neurosci **14**, 4571–87.

Goldman, S. A. (1998). Adult neurogenesis: From canaries to the clinic. J Neurobiol **36**, 267–286.

Goldman, S. A., Lemmon, V., and Chin, S. S. (1993). Migration of newly generated neurons upon ependymally derived radial guide cells in explant cultures of the adult songbird forebrain. Glia **8**, 150–160.

Goldman, S. A., and Nottebohm, F. (1983). Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proceedings of the National Academy of Sciences **80**, 2390–2394.

Gould, E., Tanapat, P., McEwen, B., Flugge, G., and Fuchs, E. (1998). Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proc. Natl. Acad. Sci. **95**, 3168–3171.

Grandpre, T., and Strittmatter, S. M. (2001). Nogo: A molecular determinant of axonal growth and regeneration. Neuroscientist **7**, 377–386.

Gravel, M., Di Polo, A., Valera, P. B., and Braun, P. E. (1998). Four-kilobase sequence of the mouse CNP gene directs spatial and temporal expression of lacZ in transgenic mice. J Neurosci Res **53**, 393–404.

Gravel, R. A., Triggs-Raine, B. L., and Mahuran, D. J. (1991). Biochemistry and genetics of Tay-Sachs disease. Can J Neurol Sci. **18**, 419–423.

Grever, W. E., Zhang, S., Ge, B., and Duncan, I. D. (1999). Fractionation and enrichment of oligodendrocytes from developing human brain. J Neurosci Res **57**, 304–14.

Grinspan, J. B., Stern, J. L., Franceschini, B., and Pleasure, D. (1993). Trophic effects of basic fibroblast growth factor (bFGF) on differentiated oligodendroglia: A mechanism for regeneration of the oligodendroglial lineage. J Neurosci Res **36**, 672–80.

Grow, J., and Barks, J. D. (2002). Pathogenesis of hypoxic-ischemic cerebral injury in the term infant: Current concepts. Clin Perinatol. **29**, 585–602.

Gumpel, M., Gout, O., Lubetzki, C., Gansmuller, A., and Baumann, N. (1989). Myelination and remyelination in the central nervous system by transplanted oligodendrocytes using the shiverer model. Discussion on the remyelinating cell population in adult mammals. Dev Neurosci 11, 132–139.

Gumpel, M., Lachapelle, F., Gansmuller, A., Baulac, M., Baron van Evercooren, A., and Baumann, N. (1987). Transplantation of human embryonic oligodendrocytes into shiverer brain. Ann NY Acad Sci 495, 71–85.

Hammang, J. P., Archer, D. R., and Duncan, I. D. (1997). Myelination following transplantation of EGF-responsive neural stem cells into a myelin-deficient environment. Exp Neurol 147, 84–95.

Hart, I. K., Richardson, W. D., Bolsover, S. R., and Raff, M. C. (1989a). PDGF and intracellular signaling in the timing of oligodendrocyte differentiation. J Cell Biol 109, 3411–3417.

Hart, I. K., Richardson, W. D., Heldin, C. H., Westermark, B., and Raff, M. C. (1989b). PDGF receptors on cells of the oligodendrocyte-type-2 astrocyte (O-2A) cell lineage. Development 105, 595–603.

Honmou, O., Felts, P. A., Waxman, S. G., and Kocsis, J. D. (1996). Restoration of normal conduction properties in demyelinated spinal cord axons in the adult rat by transplantation of exogenous Schwann cells. J Neurosci 16, 3199–3208.

Horner, P. J., Power, A. E., Kempermann, G., Kuhn, H. G., Palmer, T. D., Winkler, J., Thal, L. J., and Gage, F. H. (2000). Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. J Neurosci 20, 2218–2228.

Ibarrola, N., Mayer-Proschel, M., Rodriguez-Pena, A., and Noble, M. (1996). Evidence for the existence of at least two timing mechanisms that contribute to oligodendrocyte generation in vitro. Dev. Biol. 180, 1–21.

Imaizumi, T., Lankford, K. L., and Kocsis, J. D. (2000). Transplantation of olfactory ensheathing cells or Schwann cells restores rapid and secure conduction across the transected spinal cord. Brain Res 854, 70–78.

Imaizumi, T., Lankford, K. L., Waxman, S. G., Greer, C. A., and Kocsis, J. D. (1998). Transplanted olfactory ensheathing cells remyelinate and enhance axonal conduction in the demyelinated dorsal columns of the rat spinal cord. J Neurosci 18, 6176–6185.

Iwashita, Y., Fawcett, J. W., Crang, A. J., Franklin, R. J., and Blakemore, W. F. (2000). Schwann Cells Transplanted into Normal and X-Irradiated Adult White Matter Do Not Migrate Extensively and Show Poor Long-Term Survival. Exp Neurol 164, 292–302.

Kaplan, M. S. (1985). Formation and turnover of neurons in young and senescent animals: An electronmicroscopic and morphometric analysis. Ann NY Acad Sci 457, 173–192.

Kaplan, M. S., and Hinds, J. W. (1977). Neurogenesis in the adult rat: Electron microscopic analysis of light radioautographs. Science 197, 1092–1094.

Kato, T., Honmou, O., Uede, T., Hashi, K., and Kocsis, J. D. (2000). Transplantation of human olfactory ensheathing cells elicits remyelination of demyelinated rat spinal cord. Glia 30, 209–218.

Keirstead, H. S., Levine, J. M., and Blakemore, W. F. (1998). Response of the oligodendrocyte progenitor cell population (defined by NG2 labelling) to demyelination of the adult spinal cord. Glia 22, 161–170.

Kerr, D. A., and Ayetey, H. (2002). Immunopathogenesis of acute transverse myelitis. Curr Opin Neurol. Current Opinion in Neurology 15, 339–347.

Keyoung, H. M., Roy, N. S., Benraiss, A., Louissaint, A., Jr., Suzuki, A., Hashimoto, M., Rashbaum, W. K., Okano, H., and Goldman, S. A. (2001). High-yield selection and extraction of two promoter-defined phenotypes of neural stem cells from the fetal human brain. Nat Biotechnol 19, 843–850.

Kim, S. U., Sato, Y., Silberberg, D. H., Pleasure, D. E., and Rorke, L. B. (1983). Long-term culture of human oligodendrocytes. Isolation, growth and identification. J Neurol Sci 62, 295–301.

Kirschenbaum, B., Nedergaard, M., Preuss, A., Barami, K., Fraser, R. A., and Goldman, S. A. (1994). In vitro neuronal production and differentiation by precursor cells derived from the adult human forebrain. Cerebral Cortex 4, 576–589.

Kleinschmidt-DeMasters, B. K., and Gilden, D. H. (2001). The expanding spectrum of herpesvirus infections of the nervous system. Brain Pathol. 11, 440–451.

Koeppen, A. H., and Robitaille, Y. (2002). Pelizaeus-Merzbacher disease. J Neuropathol Exp Neurol. 61, 747–759.

Kohama, I., Lankford, K. L., Preiningerova, J., White, F. A., Vollmer, T. L., and Kocsis, J. D. (2001). Transplantation of cryopreserved adult human Schwann cells enhances axonal conduction in demyelinated spinal cord. J Neurosci 21, 944–950.

Kolter, T., and Sandhoff, K. (1998). Glycosphingolipid degradation and animal models of GM2-gangliosidoses. J Inherit Metab Dis. 21, 548–563.

Kondo, T., and Raff, M. (2000). Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. Science 289, 1754–1757.

Konstantinou, D., Paschalis, C., Maraziotis, T., Dimopoulos, P., Bassaris, H., and Skoutelis, A. (2001). Two episodes of leukoencephalitis associated with recombinant hepatitis B vaccination in a single patient. Clin Infect Dis. 33, 1772–1773.

Kotil, K., Kalayci, M., Koseoglu, T., and Tugrul, A. (2002). Myelinoclastic diffuse sclerosis (Schilder's disease): Report of a case and review of the literature. Br J Neurosurg 16, 516–519.

Kukekov, V. G., Laywell, E. D., Suslov, O., Davies, K., Scheffler, B., Thomas, L. B., O'Brien, T. F., Kusakabe, M., and Steindler, D. A. (1999). Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. Exp Neurol 156, 333–344.

Lachapelle, F., Gumpel, M., Baulac, M., Jacque, C., Duc, P., and Baumann, N. (1983). Transplantation of CNS fragments into the brain of shiverer mutant mice: Extensive myelination by implanted oligodendrocytes. I. Immunohistochemical studies. Dev Neurosci 6, 325–334.

Lakatos, A., Franklin, R. J., and Barnett, S. C. (2000). Olfactory ensheathing cells and Schwann cells differ in their in vitro interactions with astrocytes. Glia **32**, 214–225.

Learish, R. D., Brustle, O., Zhang, S. C., and Duncan, I. D. (1999). Intraventricular transplantation of oligodendrocyte progenitors into a fetal myelin mutant results in widespread formation of myelin. Ann Neurol **46**, 716–722.

Lee, S. C., Liu, W., Brosnan, C. F., and Dickson, D. W. (1992). Characterization of primary human fetal dissociated central nervous system cultures with an emphasis on microglia. Lab Invest **67**, 465–76.

Levine, J. M., and Reynolds, R. (1999). Activation and proliferation of endogenous oligodendrocyte precursor cells during ethidium bromide-induced demyelination. Exp Neurol **160**, 333–347.

Levine, J. M., Reynolds, R., and Fawcett, J. W. (2001). The oligodendrocyte precursor cell in health and disease. Trends Neurosci **24**, 39–47.

Levine, J. M., Stincone, F., and Lee, Y. S. (1993). Development and differentiation of glial precursor cells in the rat cerebellum. Glia **7**, 307–321.

Levison, S. W., and Goldman, J. E. (1993). Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain. Neuron **10**, 201–212.

Levison, S. W., Rothstein, R. P., Romanko, M. J., Snyder, M. J., Meyers, R. L., and Vannucci, S. J. (2001). Hypoxia/ischemia depletes the rat perinatal subventricular zone of oligodendrocyte progenitors and neural stem cells. Developmental Neuroscience **23**, 234–247.

Levison, S. W., Young, G. M., and Goldman, J. E. (1999). Cycling cells in the adult rat neocortex preferentially generate oligodendroglia. J Neurosci Res **57**, 435–446.

Leys, D., Englund, E., Del Se, r. T., Inzitari, D., Fazekas, F., Bornstein, N., Erkinjuntti, T., Bowler, J. V., Pantoni, L., Parnetti, L., De Reuck, J., Ferro, J., and Bogousslavsky, J. (1999). White matter changes in stroke patients. Relationship with stroke subtype and outcome. European Journal of Neurology **42**, 67–75.

Liu, S., Qu, Y., Stewart, T. J., Howard, M. J., Chakrabortty, S., Holekamp, T. F., and McDonald, J. W. (2000). Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc Natl Acad Sci USA **97**, 6126–6131.

Lois, C., and Alvarez-Buylla, A. (1993). Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proceedings of the National Academy of Sciences **90**, 2074–2077.

Luskin, M. B. (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron **11**, 173–189.

Maeda, Y., Solanky, M., Menonna, J., Chapin, J., Li, W., and Dowling, P. (2001). Platelet-derived growth factor-alpha receptor-positive oligodendroglia are frequent in multiple sclerosis lesions. Ann Neurol **49**, 776–785.

Marmur, R., Mabie, P. C., Gokhan, S., Song, Q., Kessler, J. A., and Mehler, M. F. (1998). Isolation and developmental characterization of cerebral cortical multipotent progenitors. Dev Biol **204**, 577–591.

Marusich, M., Furneaux, H., Henion, P., and Weston, J. (1994). Hu neuronal proteins are expressed in proliferating neurogenic cells. Journal of Neurobiology **25**, 143–155.

Mason, J. L., and Goldman, J. E. (2002). A2B5+ and O4+ Cycling progenitors in the adult forebrain white matter respond differentially to PDGF-AA, FGF-2, and IGF-1. Mol Cell Neurosci **20**, 30–42.

Matalon, R., Rady, P. L., Platt, K. A., Skinner, H. B., Quast, M. J., Campbell, G. A., Matalon, K., Ceci, J. D., Tyring, S. K., Nehls, M., Surendran, S., Wei, J., Ezell, E. L., and Szucs, S. (2000). Knock-out mouse for Canavan disease: A model for gene transfer to the central nervous system. J Gene Med. **2**, 165–175.

Matalon, R. M., and Michals-Matalon, K. (2000). Spongy degeneration of the brain, Canavan disease: Biochemical and molecular findings. Front Biosci. **5**, D307-D311.

McKinnon, R. D., Matsui, T., Dubois-Dalcq, M., and Aaronson, S. A. (1990). FGF modulates the PDGF-driven pathway of oligodendrocyte development. Neuron **5**, 603–614.

McMorris, F. A., and Dubois-Dalcq, M. (1988). Insulin-like growth factor I promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing in vitro. J Neurosci Res **21**, 199–209.

McTigue, D. M., Horner, P. J., Stokes, B. T., and Gage, F. H. (1998). Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. J Neurosci **18**, 5354–5365.

Mezey, E., Chandross, K. J., Harta, G., Maki, R. A., and McKercher, S. R. (2000). Turning blood into brain: Cells bearing neuronal antigens generated in vivo from bone marrow. Science **290**, 1779–1782.

Mikoshiba, K., Fujishiro, M., Kohsaka, S., and Okano, H. (1985). Takamatsu K, Tsukada Y. Disorders in myelination in the twitcher mutant: Immunohistochemical and biochemical studies. Neurochem Res. **10**, 1129–1141.

Miller, F. D., Naus, C. C., Durand, M., Bloom, F. E., and Milner, R. J. (1987). Isotypes of alpha-tubulin are differentially regulated during neuronal maturation. J Cell Biol **105**, 3065–3073.

Miller, F. D., Tetzlaff, W., Bisby, M. A., Fawcett, J. W., and Milner, R. J. (1989). Rapid induction of the major embryonic alpha-tubulin mRNA, T alpha 1, during nerve regeneration in adult rats. J Neurosci **9**, 1452–1463.

Mitome, M., Low, H. P., van Den Pol, A., Nunnari, J. J., Wolf, M. K., Billings-Gagliardi, S., and Schwartz, W. J. (2001). Towards the reconstruction of central nervous system white matter using neural precursor cells. Brain **124**, 2147–2161.

Moore, G. R., Neumann, P. E., Suzuki, K., Lijtmaer, H. N., Traugott, U., and Raine, C. S. (1985). Balo's concentric sclerosis: New observations on lesion development. Ann Neurol **17**, 604–611.

Nishiyama, A., Lin, X. H., Giese, N., Heldin, C. H., and Stallcup, W. B. (1996). Co-localization of NG2 proteoglycan and PDGF alpha-receptor on O2A progenitor cells in the developing rat brain. J Neurosci Res **43**, 299–314.

Nishiyama, A., Yu, M., Drazba, J. A., and Tuohy, V. K. (1997). Normal and reactive NG2+ glial cells are distinct from resting and activated microglia. J Neurosci Res **48**, 299–312.

Noble, M. (1997). The oligodendrocyte-type 2 astrocyte lineage: In vitro and in vivo studies on development, tissue repair and neoplasia. *In* "Isolation, Characterization and Utilization of CNS Stem Cells" (Y. C. F. Gage, ed.), pp. 101–128. Springer-Verlag, Berlin.

Noble, M., Murray, K., Stroobant, P., Waterfield, M. D., and Riddle, P. (1988). Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell. Nature **333**, 560–562.

Noble, M., Wren, D., and Wolswijk, G. (1992). The O-2A(adult) progenitor cell: A glial stem cell of the adult central nervous system. Semin Cell Biol **3**, 413–422.

Nottebohm, F. (1985). Neuronal replacement in adulthood. Ann NY Acad Sci **457**, 143–161.

Nunes, M. C., Roy, N. S., Keyoung, H. M., Goodman, R., McKhann, G., Kang, J., Jiang, L., Nedergaard, M., and Goldman, S. (2003). Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. Nature Med. **9**, 439–447.

O'Neill, R. C., Minuk, J., Cox, M. E., Braun, P. E., and Gravel, M. (1997). CNP2 mRNA directs synthesis of both CNP1 and CNP2 polypeptides. J Neurosci Res **50**, 248–257.

Palmer, T. D., Ray, J., and Gage, F. H. (1995). FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol Cell Neurosci **6**, 474–486.

Pincus, D. W., Keyoung, H. M., Harrison-Restelli, C., Goodman, R. R., Fraser, R. A., Edgar, M., Sakakibara, S., Okano, H., Nedergaard, M., and Goldman, S. A. (1998). Fibroblast growth factor-2/brain-derived neurotrophic factor-associated maturation of new neurons generated from adult human subependymal cells. Ann Neurol **43**, 576–585.

Pouly, S., Becher, B., Blain, M., and Antel, J. P. (1999). Expression of a homologue of rat NG2 on human microglia. Glia **27**, 259–268.

Prabhakar, S., D'Souza, S., Antel, J. P., McLaurin, J., Schipper, H. M., and Wang, E. (1995). Phenotypic and cell cycle properties of human oligodendrocytes in vitro. Brain Res **672**, 159–169.

Prineas, J. W., and Connell, F. (1979). Remyelination in multiple sclerosis. Ann Neurol **5**, 22–31.

Prineas, J. W., Kwon, E. E., Cho, E. S., and Sharer, L. R. (1984). Continual breakdown and regeneration of myelin in progressive multiple sclerosis plaques. Ann NY Acad Sci **436**, 11–32.

Prineas, J. W., Kwon, E. E., Goldenberg, P. Z., Ilyas, A. A., Quarles, R. H., Benjamins, J. A., and Sprinkle, T. J. (1989). Multiple sclerosis. Oligodendrocyte proliferation and differentiation in fresh lesions. Lab Invest **61**, 489–503.

Pringle, N. P., Mudhar, H. S., Collarini, E. J., and Richardson, W. D. (1992). PDGF receptors in the rat CNS: During late neurogenesis, PDGF alpha-receptor expression appears to be restricted to glial cells of the oligodendrocyte lineage. Development **115**, 535–551.

Qian, X., Davis, A. A., Goderie, S. K., and Temple, S. (1997). FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. Neuron **18**, 81–93.

Raabe, T. D., Clive, D. R., Wen, D., and DeVries, G. H. (1997). Neonatal oligodendrocytes contain and secrete neuregulins in vitro. J Neurochem **69**, 1859–1863.

Raff, M. C., Abney, E. R., Cohen, J., Lindsay, R., and Noble, M. (1983a). Two types of astrocytes in cultures of developing rat white matter: Differences in morphology, surface gangliosides, and growth characteristics. J Neurosci **3**, 1289–1300.

Raff, M. C., Lillien, L. E., Richardson, W. D., Burne, J. F., and Noble, M. D. (1988). Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. Nature **333**, 562–565.

Raff, M. C., Miller, R. H., and Noble, M. (1983b). Glial cell lineages in the rat optic nerve. Cold Spring Harb Symp Quant Biol **48**, 569–572.

Rakic, S., and Zecevic, N. (2003). Early oligodendrocyte progenitor cells in the human fetal telencephalon. Glia **41**, 117–127.

Ramon-Cueto, A., Plant, G. W., Avila, J., and Bunge, M. B. (1998). Long-distance axonal regeneration in the transected adult rat spinal cord is promoted by olfactory ensheathing glia transplants. J Neurosci **18**, 3803–3815.

Redwine, J. M., and Armstrong, R. C. (1998). In vivo proliferation of oligodendrocyte progenitors expressing PDGFalphaR during early remyelination. J Neurobiol **37**, 413–428.

Reubinoff, B. E., Itsykson, P., Turetsky, T., Pera, M. F., Reinhartz, E., Itzik, A., and Ben-Hur, T. (2001). Neural progenitors from human embryonic stem cells. Nat Biotechnol **19**, 1134–1140.

Reynolds, R., and Hardy, R. (1997). Oligodendroglial progenitors labeled with the O4 antibody persist in the adult rat cerebral cortex in vivo. J Neurosci Res **47**, 455–470.

Rezaie, P., and Dean, A. (2002). Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. Neuropathology **22**, 106–132.

Richards, L. J., Kilpatrick, T. J., and Bartlett, P. F. (1992). De novo generation of neuronal cells from the adult mouse brain. Proceedings of the National Academy of Sciences **89**, 8591–8595.

Roach, A., Takahashi, N., Pravtcheva, D., Ruddle, F., and Hood, L. (1985). Chromosomal mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in shiverer mutant mice. Cell **42**, 149–155.

Roy, N. S., Benraiss, A., Wang, S., Fraser, R. A., Goodman, R., Couldwell, W. T., Nedergaard, M., Kawaguchi, A., Okano, H., and Goldman, S. A. (2000a). Promoter-targeted selection and isolation of neural progenitor cells from the adult human ventricular zone. J Neurosci Res **59**, 321–331.

Roy, N. S., Wang, S., Harrison-Restelli, C., Benraiss, A., Fraser, R. A., Gravel, M., Braun, P. E., and Goldman, S. A. (1999). Identification, isolation, and promoter-defined separation of mitotic oligodendrocyte progenitor cells from the adult human subcortical white matter. J Neurosci **19**, 9986–9995.

Roy, N. S., Wang, S., Jiang, L., Kang, J., Benraiss, A., Harrison-Restelli, C., Fraser, R. A., Couldwell, W. T., Kawaguchi, A., Okano, H., Nedergaard, M., and Goldman, S. A. (2000b). In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus. Nat Med **6**, 271–277.

Rust, R. (2000). Multiple Sclerosis, acute disseminated encephalomyelitis and related conditions. *Semin. Pediatr. Neurol.* **7**, 66–90.

Rutkowski, J. L., Kirk, C. J., Lerner, M. A., and Tennekoon, G. I. (1995). Purification and expansion of human Schwann cells in vitro. Nat Med **1**, 80–83.

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., Freeman, T. B., Saporta, S., Janssen, W., Patel, N., Cooper, D. R., and Sanberg, P. R. (2000). Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol **164**, 247–256.

Saneto, R. P., Low, K. G., Melner, M. H., and de Vellis, J. (1988). Insulin/insulin-like growth factor I and other epigenetic modulators of myelin basic protein expression in isolated oligodendrocyte progenitor cells. J Neurosci Res **21**, 210–219.

Satoh, J. I., Tai, T., and Kim, S. U. (1996). Differential expression of gangliosides and galactolipids in fetal human oligodendrocytes and astrocytes in culture. Brain Res Dev Brain Res **93**, 172–181.

Scherer, S. S., Braun, P. E., Grinspan, J., Collarini, E., Wang, D. Y., and Kamholz, J. (1994). Differential regulation of the 2',3'-cyclic nucleotide 3'-phosphodiesterase gene during oligodendrocyte development. Neuron **12**, 1363–1375.

Schiffmann, R., and Boespflug-Tanguy, O. (2001). An update on the leukodsytrophies. Current Opinion in Neurology **14**, 789–794.

Scolding, N., Franklin, R., Stevens, S., Heldin, C. H., Compston, A., and Newcombe, J. (1998). Oligodendrocyte progenitors are present in the normal adult human CNS and in the lesions of multiple sclerosis. Brain **121**, 2221–2228.

Scolding, N. J., Rayner, P. J., and Compston, D. A. (1999). Identification of A2B5-positive putative oligodendrocyte progenitor cells and A2B5-positive astrocytes in adult human white matter. Neuroscience **89**, 1–4.

Scolding, N. J., Rayner, P. J., Sussman, J., Shaw, C., and Compston, D. A. (1995). A proliferative adult human oligodendrocyte progenitor. Neuroreport **6**, 441–445.

Seaberg, R. M., and van der Kooy, D. (2002). Adult rodent neurogenic regions: The ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. J Neurosci **22**, 1784–1793.

Shi, J., Marinovitch, A., and Barres, B. (1998). Purification and characterization of adult oligodendrocyte precursor cells from the rat optic nerve. J. Neurosci. **18**, 4627–4636.

Shields, S. A., Blakemore, W. F., and Franklin, R. J. (2000). Schwann cell remyelination is restricted to astrocyte-deficient areas after transplantation into demyelinated adult rat brain. J Neurosci Res **60**, 571–578.

Sim, F. J., Zhao, C., Penderis, J., and Franklin, R. J. (2002). The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. J Neurosci **22**, 2451–2459.

Smith, P. M., and Blakemore, W. F. (2000). Porcine neural progenitors require commitment to the oligodendrocyte lineage prior to transplantation in order to achieve significant remyelination of demyelinated lesions in the adult CNS. Eur J Neurosci **12**, 2414–2424.

Sprinkle, T. J. (1989). 2',3'-cyclic nucleotide 3'-phosphodiesterase, an oligodendrocyte-Schwann cell and myelin-associated enzyme of the nervous system. Crit Rev Neurobiol **4**, 235–301.

Tang, D. G., Tokumoto, Y. M., Apperly, J. A., Lloyd, A. C., and Raff, M. C. (2001). Lack of replicative senescence in cultured rat oligodendrocyte precursor cells. Science **291**, 868–871.

Tang, D. G., Tokumoto, Y. M., and Raff, M. C. (2000). Long-term culture of purified postnatal oligodendrocyte precursor cells. Evidence for an intrinsic maturation program that plays out over months. J Cell Biol **148**, 971–984.

Targett, M. P., Sussman, J., Scolding, N., O'Leary, M. T., Compston, D. A., and Blakemore, W. F. (1996). Failure to achieve remyelination of demyelinated rat axons following transplantation of glial cells obtained from the adult human brain. Neuropathol Appl Neurobiol **22**, 199–206.

Tate, B., Bower, K., and Snyder, E. (2001). Transplant herapy. *In* "Stem Cells and CNS Development" (M. Rao, ed.), pp. 291–306. Humana, Totowa, NJ.

Temple, S., and Raff, M. C. (1986). Clonal analysis of oligodendrocyte development in culture: Evidence for a developmental clock that counts cell divisions. Cell **44**, 773–739.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., Meyer, E. M., Morel, L., Petersen, B. E., and Scott, E. W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature **416**, 542–545.

Uchida, N., Buck, D. W., He, D., Reitsma, M. J., Masek, M., Phan, T. V., Tsukamoto, A. S., Gage, F. H., and Weissman, I. L. (2000). Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci USA **97**, 14720–14725.

Vartanian, T., Goodearl, A., Viehover, A., and Fischbach, G. (1997). Axonal neuregulin signals cells of the oligodendrocyte lineage through activation of HER4 and Schwann cells through HER2 and HER3. J Cell Biol 137, 211–220.

Vaughn, J. E. (1969). An electron microscopic analysis of gliogenesis in rat optic nerves. Z Zellforsch Mikrosk Anat. 94, 293–324.

Vaughn, J. E., and Peters, A. (1968). A third neuroglial cell type. An electron microscopic study. J Comp Neurol 133, 269–288.

Vitry, S., Avellana-Adalid, V., Hardy, R., Lachapelle, F., and Baron-Van Evercooren, A. (1999). Mouse oligospheres: From pre-progenitors to functional oligodendrocytes. J Neurosci Res 58, 735–751.

Vogel, U. S., and Thompson, R. J. (1988). Molecular structure, localization, and possible functions of the myelin-associated enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase. J Neurochem 50, 1667–1677.

Volpe, J. J. (2001). Neurobiology of periventricular leukomalacia in the premature infant. Pediatr Res. 50, 553–562.

Wang, S., Wu, H., Jiang, J., Delohery, T. M., Isdell, F., and Goldman, S. A. (1998). Isolation of neuronal precursors by sorting embryonic forebrain transfected with GFP regulated by the T alpha 1 tubulin promoter. Nature Biotechnol 16, 196–201.

Warrington, A. E., Barbarese, E., and Pfeiffer, S. E. (1993). Differential myelinogenic capacity of specific developmental stages of the oligodendrocyte lineage upon transplantation into hypomyelinating hosts. J Neurosci Res 34, 1–13.

Weissbarth, S., Maker, H. S., Raes, I., Brannan, T. S., Lapin, E. P., and Lehrer, G. M. (1981). The activity of 2',3'-cyclic nucleotide 3'-phosphodiesterase in rat tissues. J Neurochem 37, 677–680.

Wenger, D. A., Rafi, M. A., Luzi, P., and Datto, J. (2000). Costantino-Ceccarini E. Krabbe disease: Genetic aspects and progress toward therapy. Mol Genet Metab. 70, 1–9.

Werner, H., Jung, M., Klugmann, M., Sereda, M., Griffiths, I. R., and Nave, K. A. (1998). Mouse models of myelin diseases. Brain Pathol 8, 771–793.

Williams, B. P., Read, J., and Price, J. (1991). The generation of neurons and oligodendrocytes from a common precursor cell. Neuron 7, 685–693.

Windrem, M., Roy, N., Wang, J., Nunes, M., Benraiss, A., Goodman, R., McKhann, G., and Goldman, S. A. (2002). Progenitor cells derived from the adult human subcortical white matter disperse and differentiate as oligodendrocytes within demyelinated regions of the rat brain. J. Neurosci. Res. 69, 966–975.

Wolswijk, G., Munro, P. M., Riddle, P. N., and Noble, M. (1991). Origin, growth factor responses, and ultrastructural characteristics of an adult-specific glial progenitor cell. Ann NY Acad Sci 633, 502–504.

Wolswijk, G., and Noble, M. (1989). Identification of an adult-specific glial progenitor cell. Development 105, 387–400.

Wolswijk, G., Riddle, P. N., and Noble, M. (1990). Coexistence of perinatal and adult forms of a glial progenitor cell during development of the rat optic nerve. Development 109, 691–698.

Wren, D., Wolswijk, G., and Noble, M. (1992). In vitro analysis of the origin and maintenance of O-2Aadult progenitor cells. J Cell Biol 116, 167–176.

Wu, E., and Raine, C. S. (1992). Multiple sclerosis. Interactions between oligodendrocytes and hypertrophic astrocytes and their occurrence in other, nondemyelinating conditions. Lab Invest 67, 88–99.

Yan, H., and Wood, P. M. (2000). NT-3 weakly stimulates proliferation of adult rat O1(−)O4(+) oligodendrocyte-lineage cells and increases oligodendrocyte myelination in vitro. J Neurosci Res 62, 329–335.

Yandava, B., Billinghurst, L., and Snyder, E. (1999). Global cell replacement is feasible via neural stem cell transplantation: Evidence from the dysmyelinated shiverer mouse brain. Proc. Natl. Acad. Sci. 96, 7029–7034.

Ying, Q. L., Nichols, J., Evans, E. P., and Smith, A. G. (2002). Changing potency by spontaneous fusion. Nature 416, 545–548.

Yoshimura, T., Kobayashi, T., Mitsuo, K., and Goto, I. (1989). Decreased fatty acylation of myelin proteolipid protein in the twitcher mouse. J Neurochemistry 52, 836–841.

Yu, W. P., Collarini, E. J., Pringle, N. P., and Richardson, W. D. (1994). Embryonic expression of myelin genes: Evidence for a focal source of oligodendrocyte precursors in the ventricular zone of the neural tube. Neuron 12, 1353–1362.

Zhang, S. C., Ge, B., and Duncan, I. D. (1999). Adult brain retains the potential to generate oligodendroglial progenitors with extensive myelination capacity. Proc Natl Acad Sci USA 96, 4089–4094.

Zhang, S. C., Ge, B., and Duncan, I. D. (2000). Tracing human oligodendroglial development in vitro. J Neurosci Res 59, 421–429.

Zhang, S. C., Lundberg, C., Lipsitz, D., O'Connor, L. T., and Duncan, I. D. (1998). Generation of oligodendroglial progenitors from neural stem cells. J Neurocytol 27, 475–489.